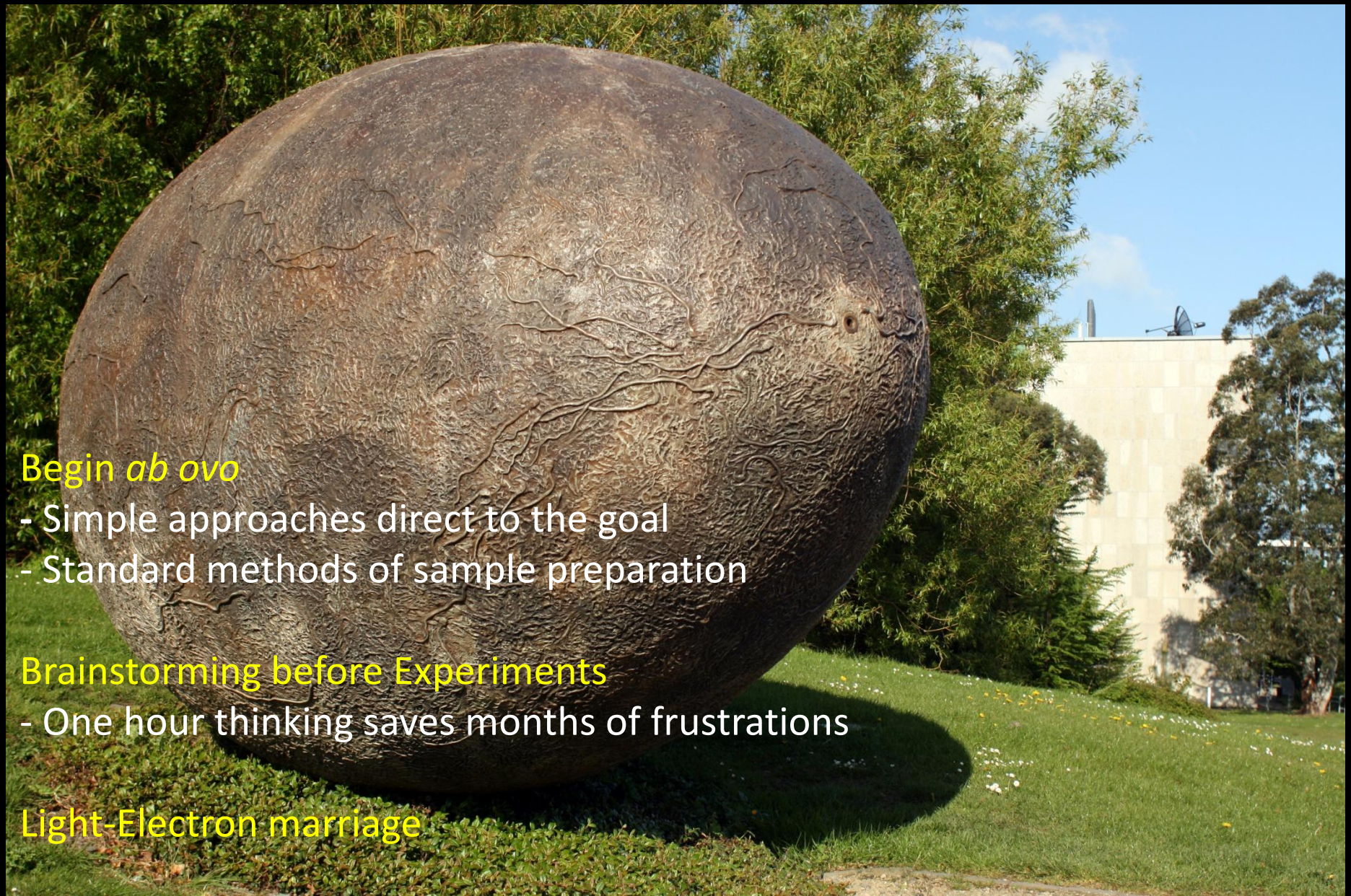
EUV microscopy - a user's perspective

Dimitri Scholz EUV, 09.11.2011



Begin *ab ovo*

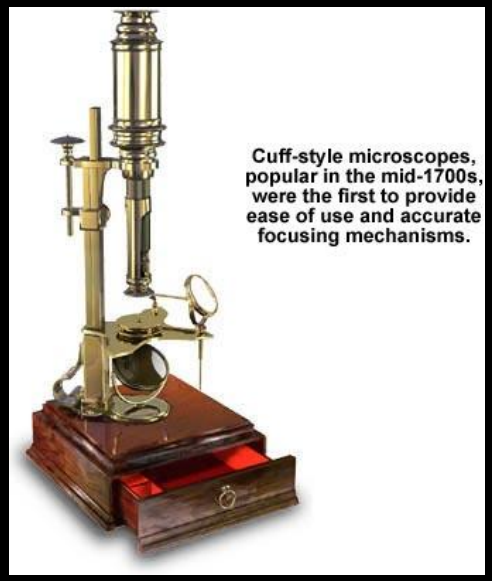
- Simple approaches direct to the goal
- Standard methods of sample preparation

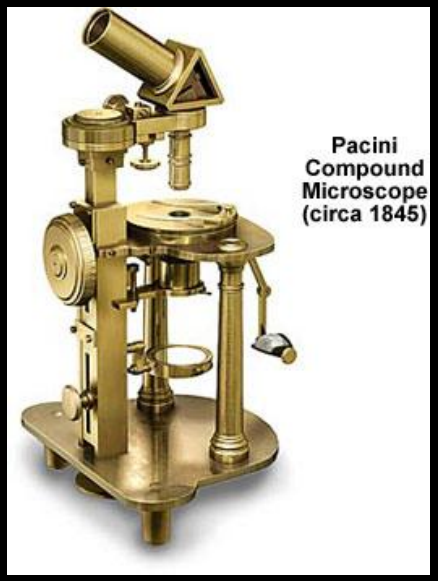
Brainstorming before Experiments

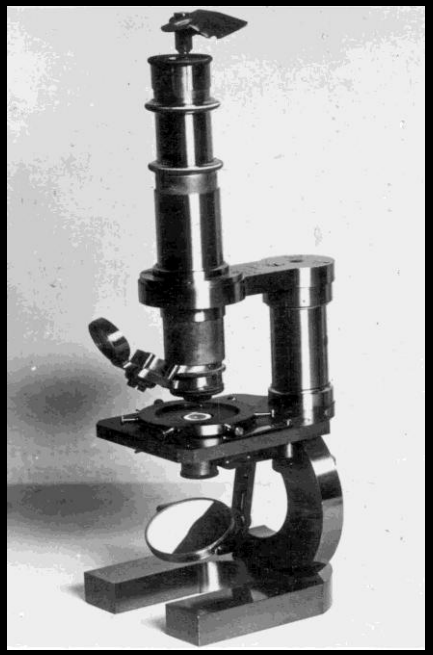
- One hour thinking saves months of frustrations

Light-Electron marriage

Glorious history of microscopy







Make me a confocal picture!

Electron microscopy

Light microscopy

Transmission light

Fluorescent light

Bright field

Polarized light

Nomarski contrast (DIC)

Epi-fluorescent

Confocal

TIRF

SPIM

Structured Illumination

Two-Photon

Decon-volution

Single pinhole

Parallel array

Spinning disc

Total Internal Reflection Fluorescence

Single Plane Illumination Microscopy

Electron microscopy

Light microscopy

TEM

Biomed

W or LaB6 cathode

80 kEV

Resolution 1 nm

Materials

FEG cathode

200-300 kEV

Resolution 0.1 nm

SEM

Table - top

W cathode

5-30 kEV

Resolution ca. 5-20 nm

BSE

E_2

High spec

FEG cathode

80-300 kEV

Resolution ca. 1 nm

E_2

Environ mental

Low vac

FIB

X-ray

Fluor

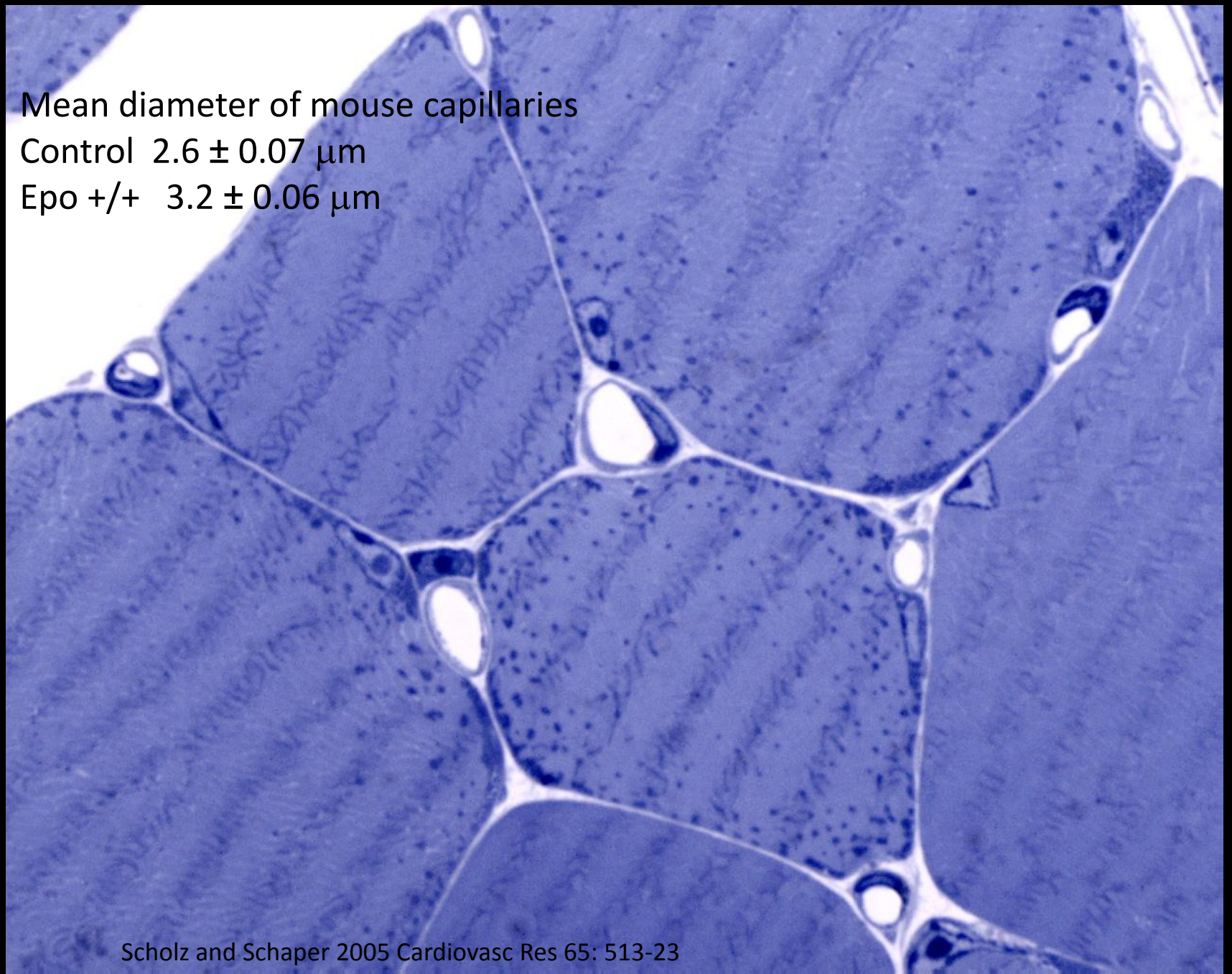
BSE

EDAX

Frozen

Please no Eierliegende Wollmilchsau!

Transmission light microscopy

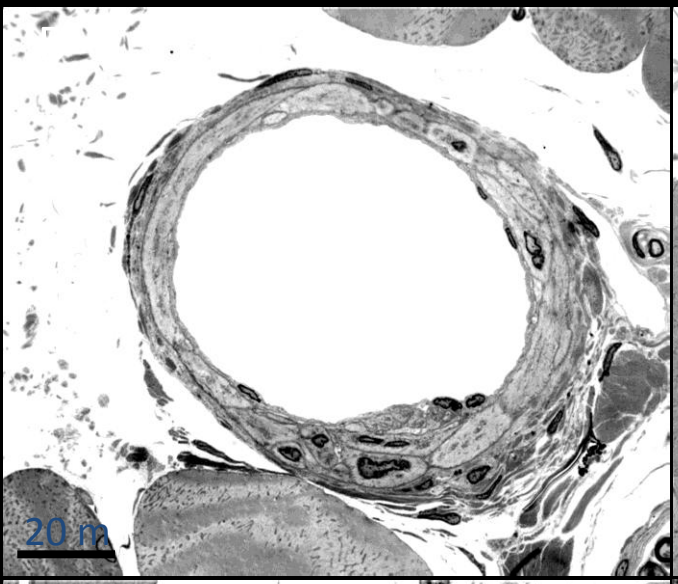


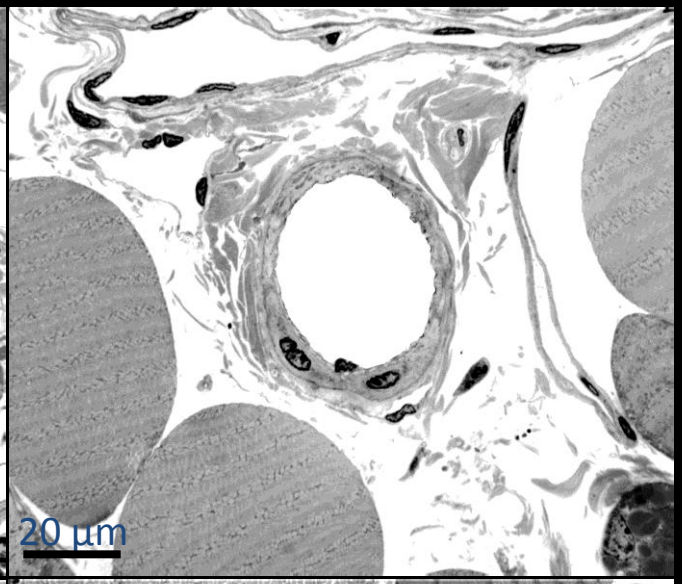
Transmission light microscopy

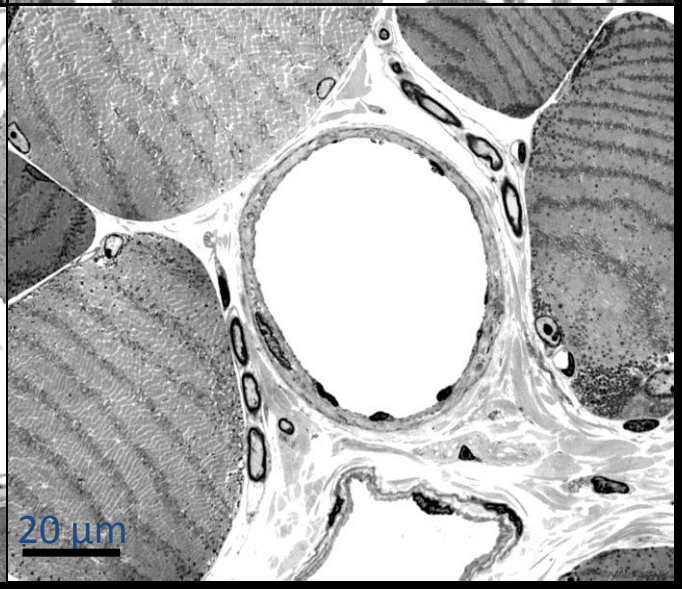
Growth of collateral arteries continues under zero shear stress

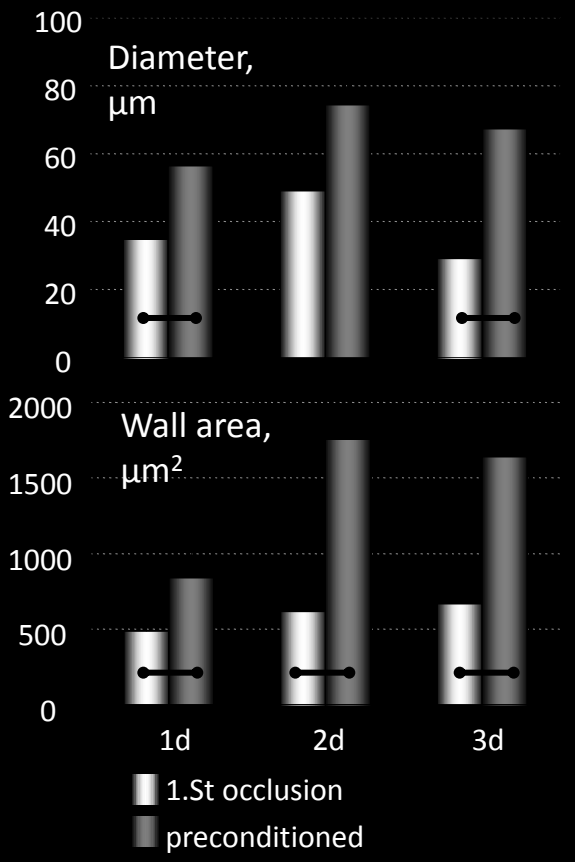
3d ischemia+14d reperfusion

3d ischemia



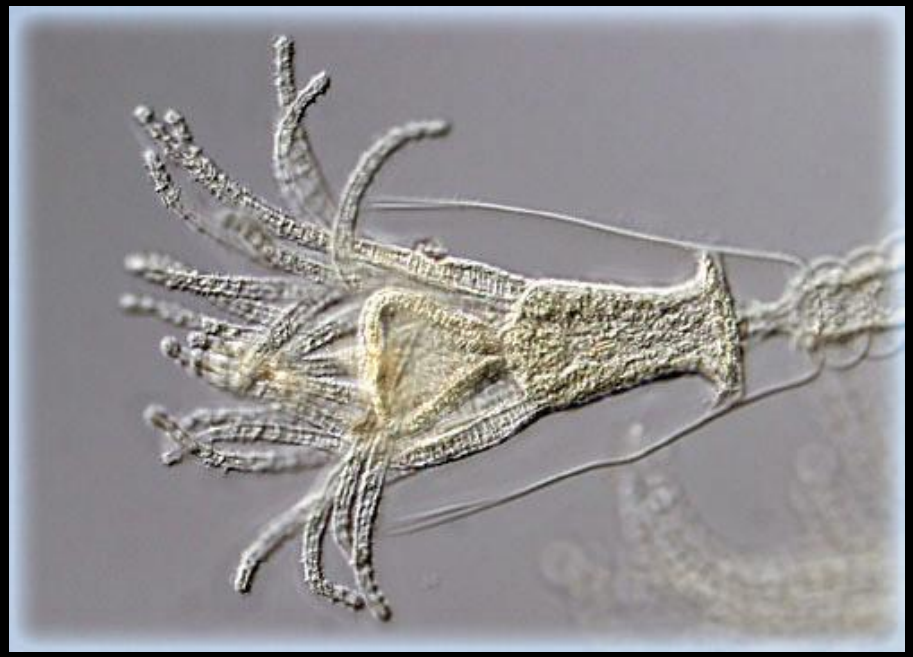






Scholz and Schaper 2005 Cardiovasc Res 65: 513-23

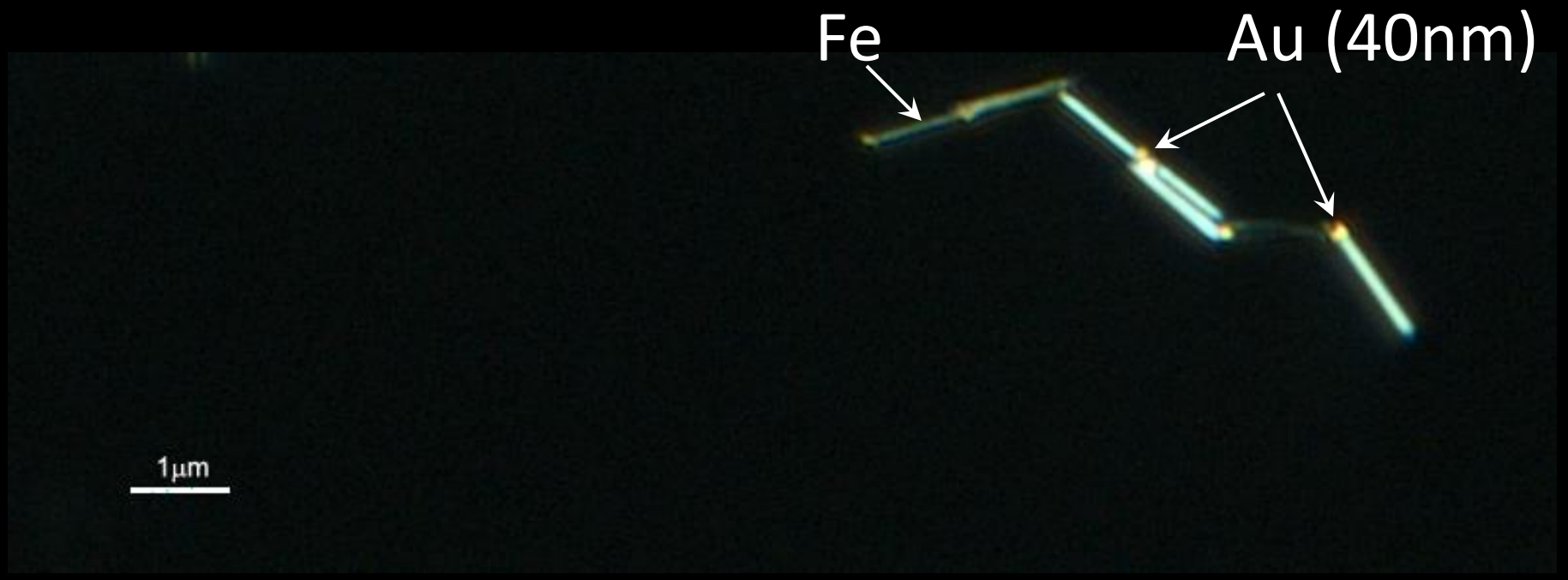
Polarized light, DIC microscopy



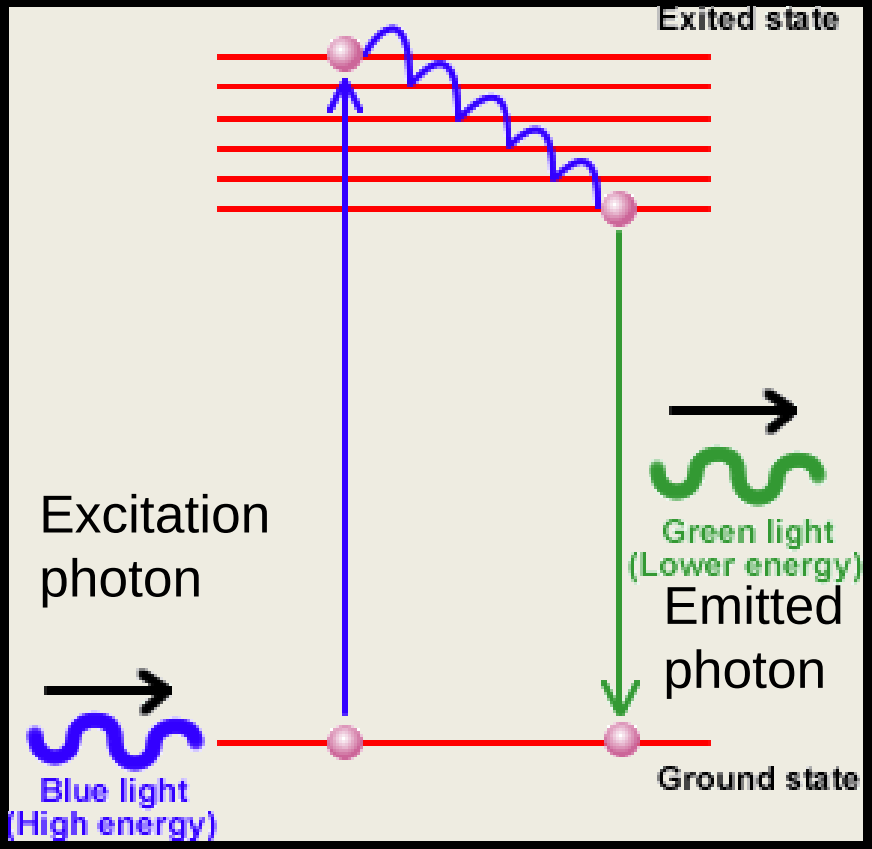
Transmission light microscopy, DIC

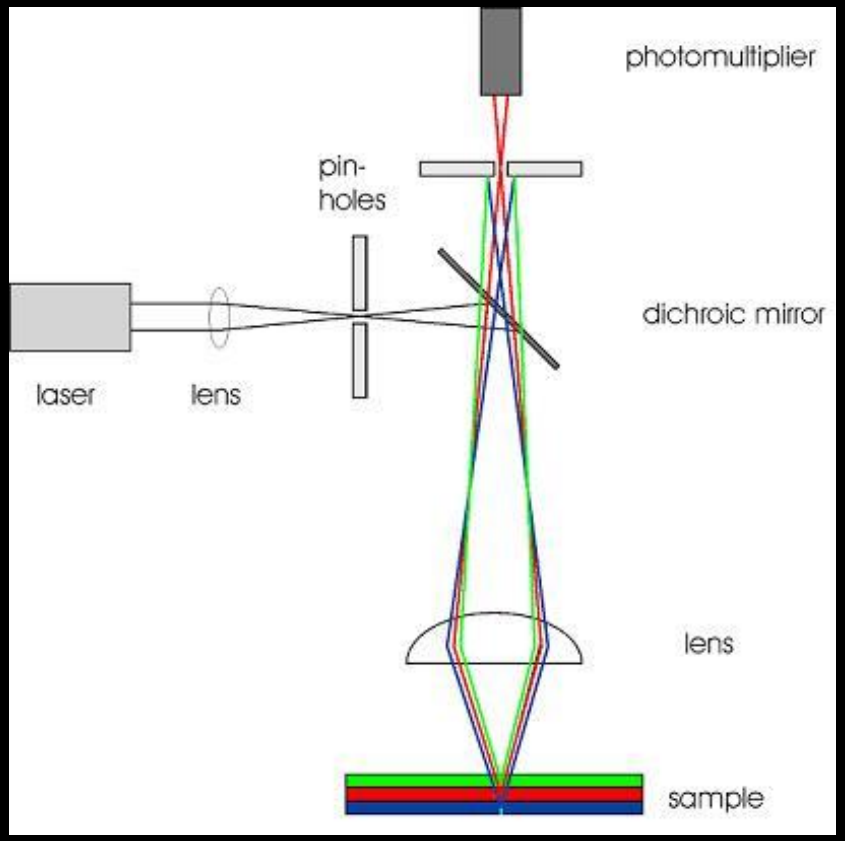
Fe/Au Nanoparticles ca. 1 μm

Polarised Light
Condenser 0il NA 1.4
Objective 100x TIRF NA 1.49
Best possible resolution 2011

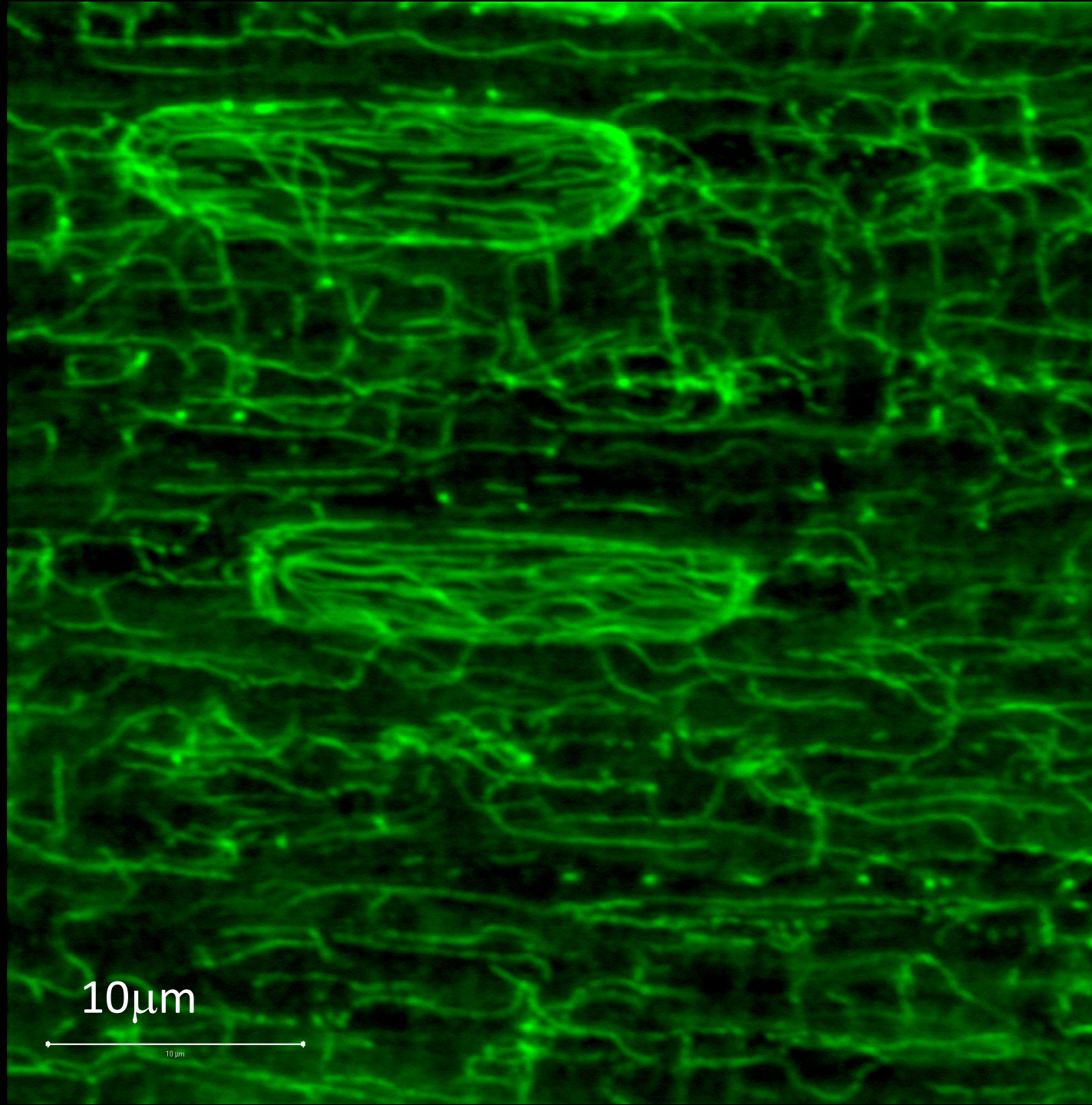


Fluorescence ≠ Confocal





Microtubules: Imunolabeling against α-Tubulin



Scholz et al, AJP 294: H1135-44 (2008)

3D-Reconstruction of ca. 100 confocal optical Sections

Zebrafish retina

FLUOVIEW® FV10i

Low spec 2009 > High spec 1999

Image Resolution

Resolution: the minimum separation (s) necessary between two point objects in the sample so that they can be distinguished as separate

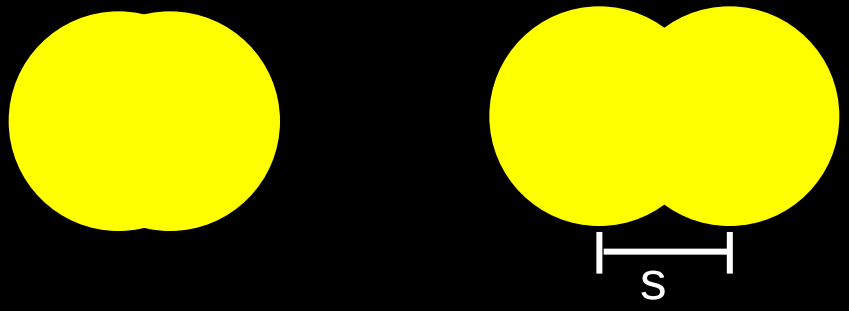
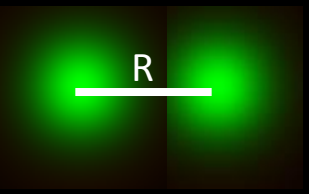


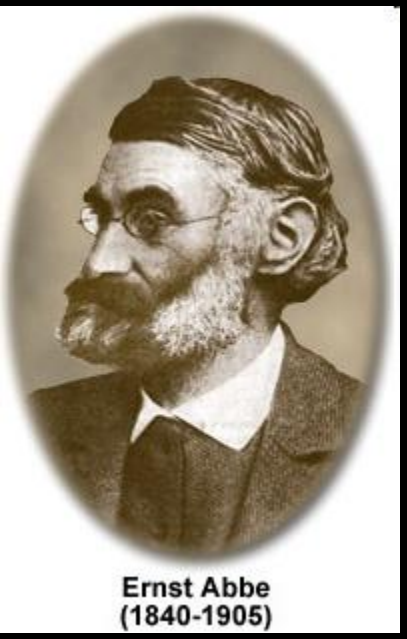
Image Resolution is limited by two factors:

1. Optical Resolution
2. Detector Resolution

Spatial (XY) resolution



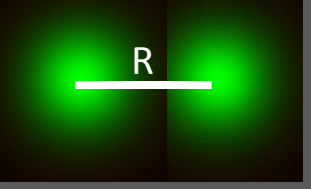
Spatial resolution = the minimal distance between two objects to recognize them as separated



Founder of modern microscopy

$$R = \frac{1.22\,\lambda}{NA_{obj} + NA_{cond}} \approx \frac{0.61\,\lambda}{NA} \sim \frac{\lambda}{2}$$

Diffractional resolution limit: point objects are detected as point spread functions (PSF)



Calculated XY resolution for popular objectives


Ernst Abbe
(1840-1905)

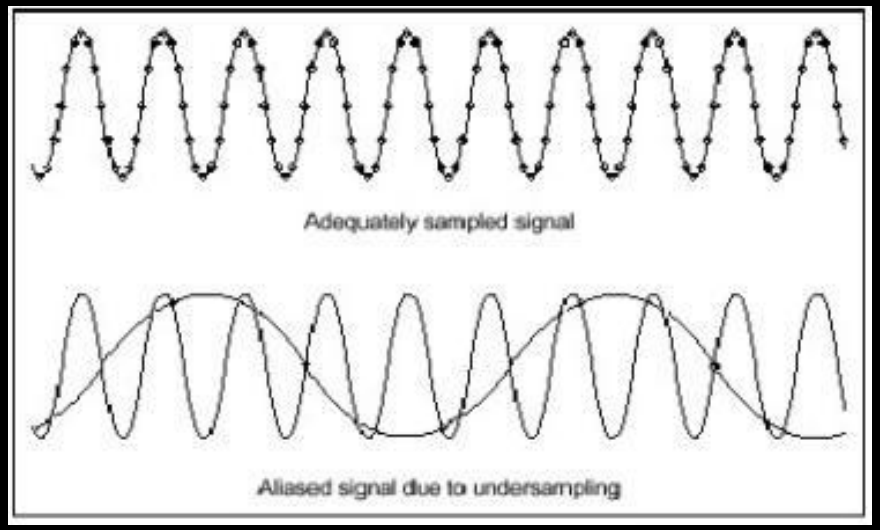
$$R = \frac{0.61\,\lambda}{NA}$$

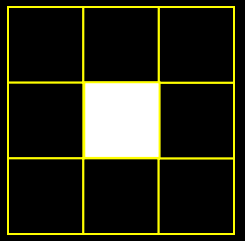
Lens	NA	400 nm	500 nm	600 nm	700nm
10	0.3	813	1017	1220	1423
10	0.40	610	763	915	1068
20	0.50	488	610	732	854
20	0.75	325	407	488	569
40	0.60	407	508	610	712
40	0.75	325	407	488	569
40	0.95	257	321	385	449
40	1.00	244	305	366	427
40	1.30	188	235	282	328
63	1.20	203	254	305	356
63	1.40	174	218	261	305
100	1.40	174	218	261	305
100	1.49	164	205	246	287

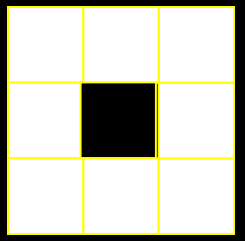
Resolution limitation by detector

Nyquist–Shannon sampling theorem:

Converting from an analog signal (sound or image) to digital, the sampling frequency must be greater than twice the highest frequency of the input signal in order to be able to reconstruct the original perfectly from the sampled version.







3:1

Agreed:
2.4:1

Not number of pixels, but their size, defines resolution

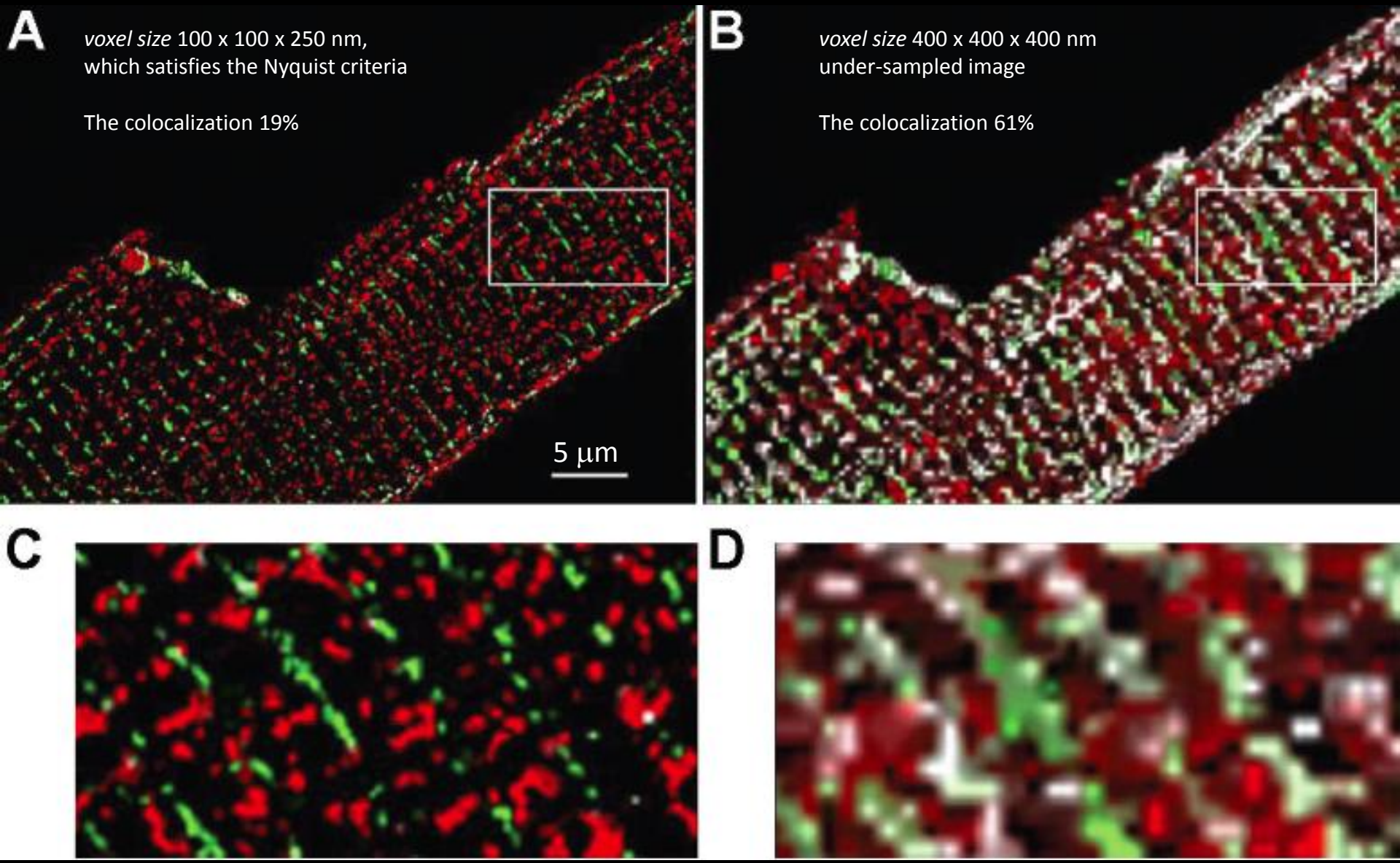
Digital camera pixel size

Lens	NA	500 nm	2x	3.4 µm	6.45 µm	8 µm	13 µm	16 µm
10	0.30	1017	508	340	645	800	1300	1600
10	0.40	763	381	340	645	800	1300	1600
20	0.50	610	305	170	323	400	650	800
20	0.75	407	203	170	323	400	650	800
40	0.60	508	254	85	161	200	325	400
40	0.75	407	203	85	161	200	325	400
40	0.95	321	161	85	161	200	325	400
40	1.00	305	153	85	161	200	325	400
40	1.30	235	117	85	161	200	325	400
63	1.20	254	127	54	102	127	206	254
63	1.40	218	109	54	102	127	206	254
100	1.40	218	109	34	65	80	130	160
100	1.49	205	102	34	65	80	130	160

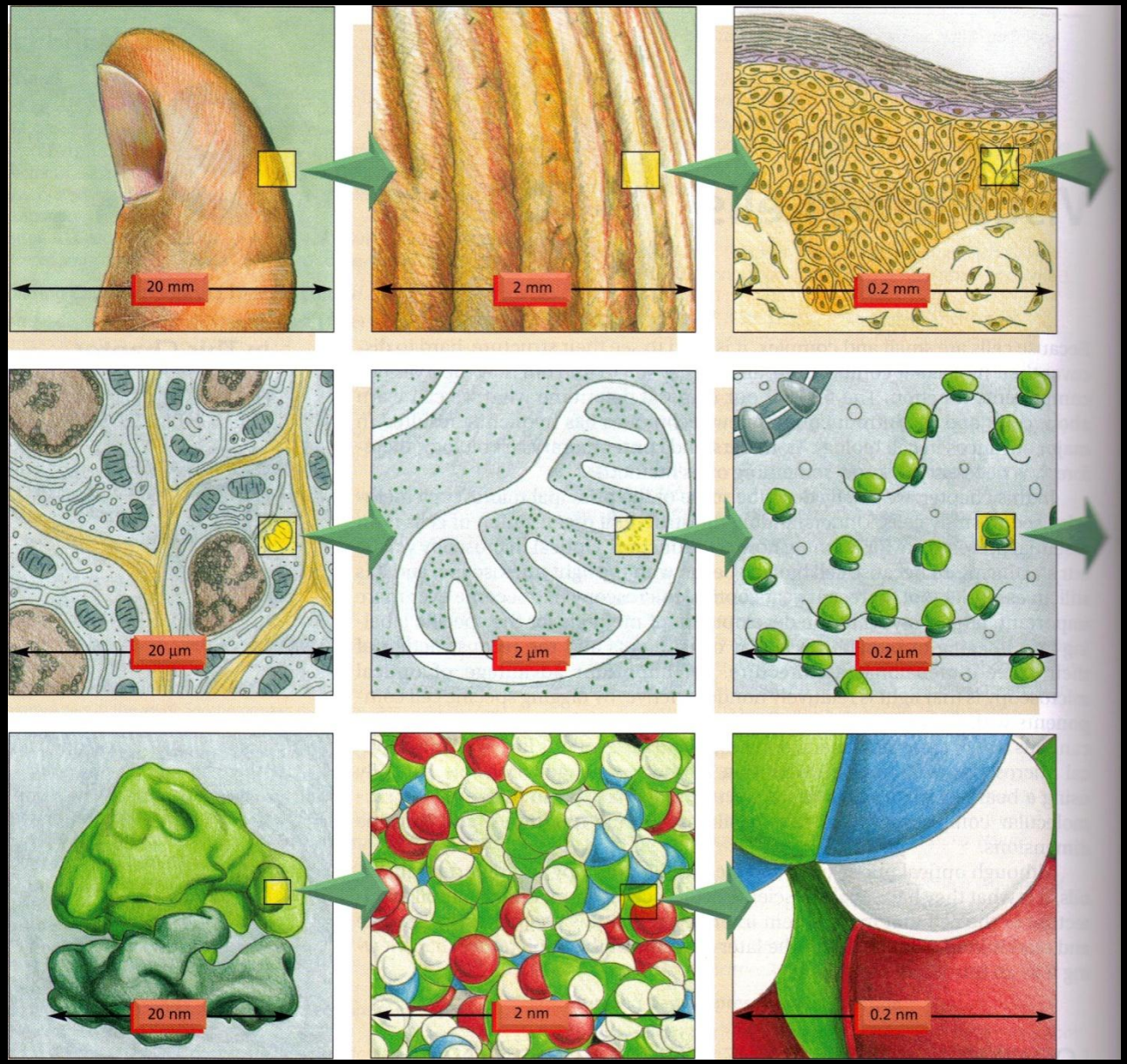
Paradox: High resolution detection is more important for low power objectives.

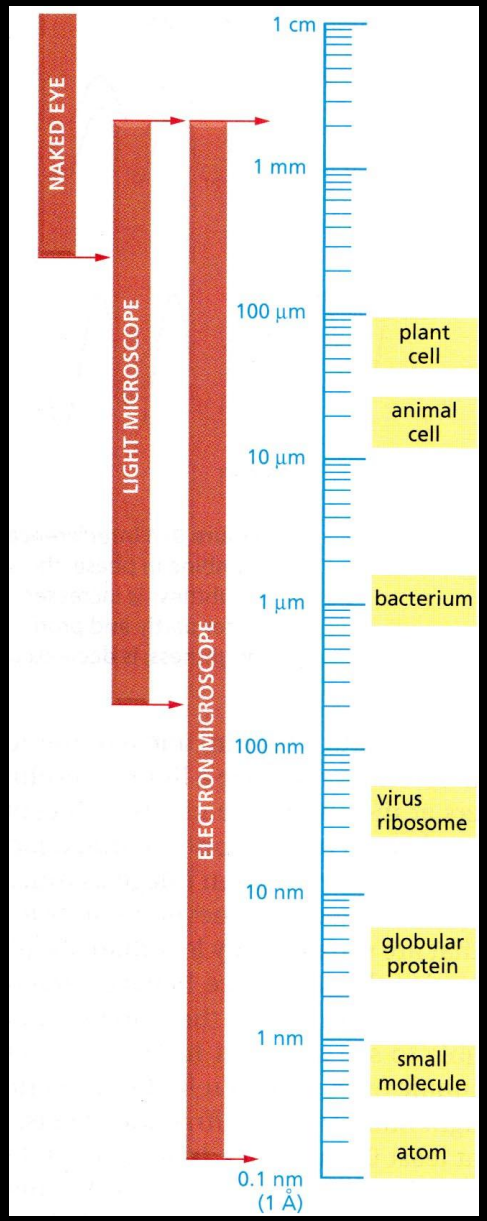
http://support.svi.nl/wiki/NyquistCalculator

Rat ventricular myocyte labeled for vinculin (green) and caveolin-3 (red), with colocalized voxels white

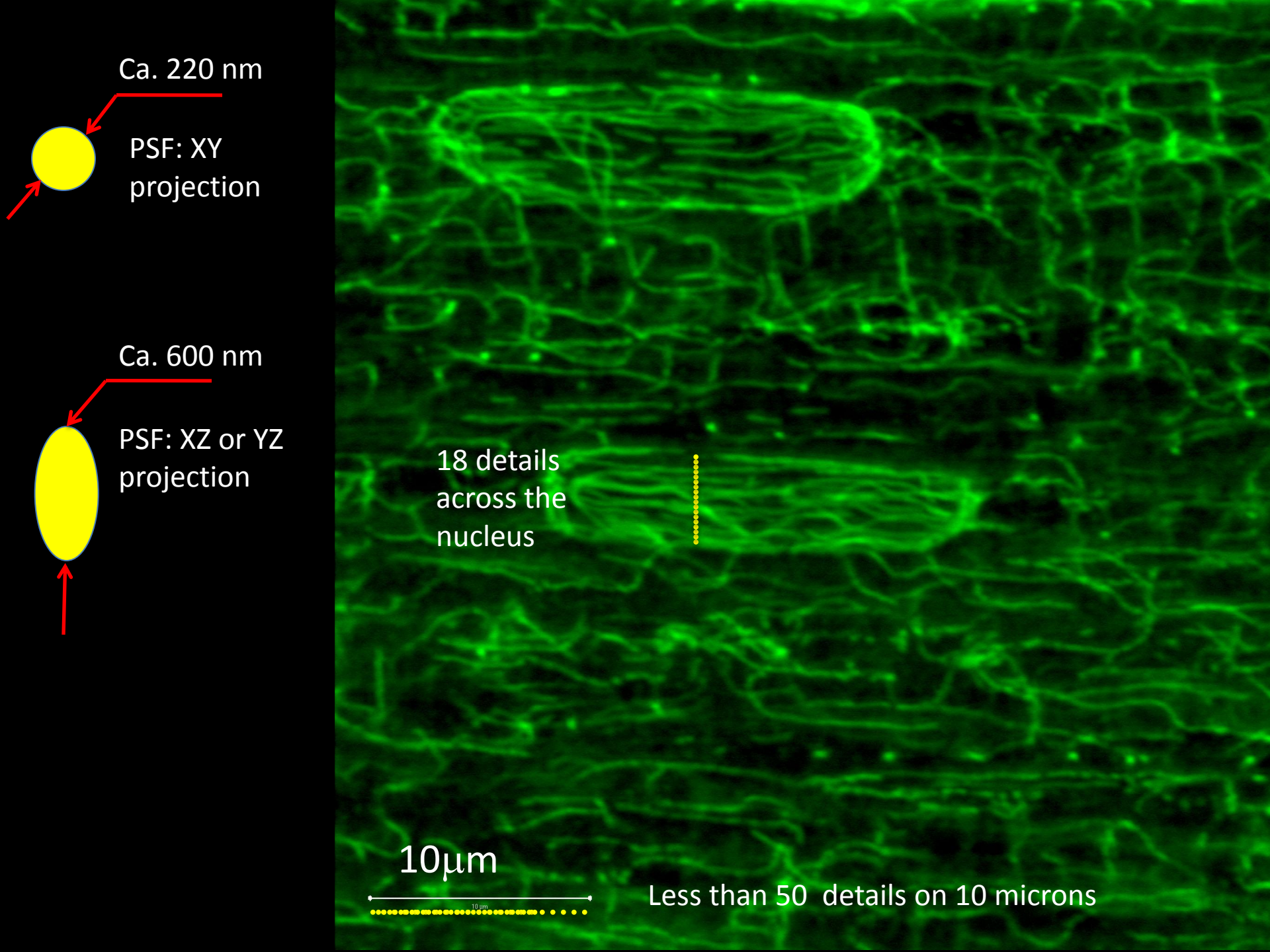


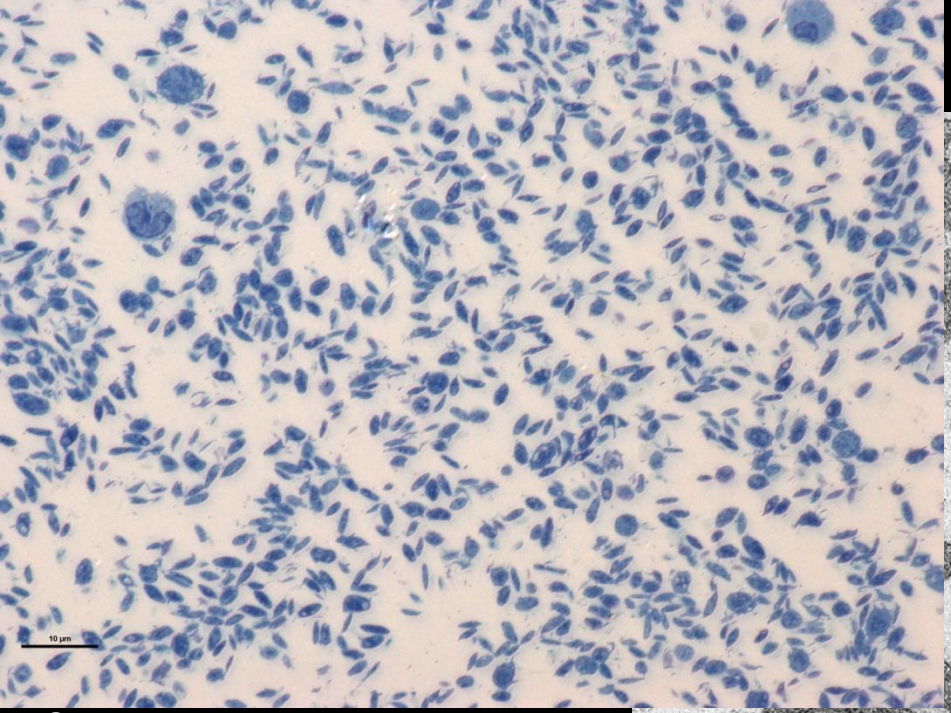
Metric scale and the ultrastructure





From Molecular Biology of Cell. 4th or 5th edition

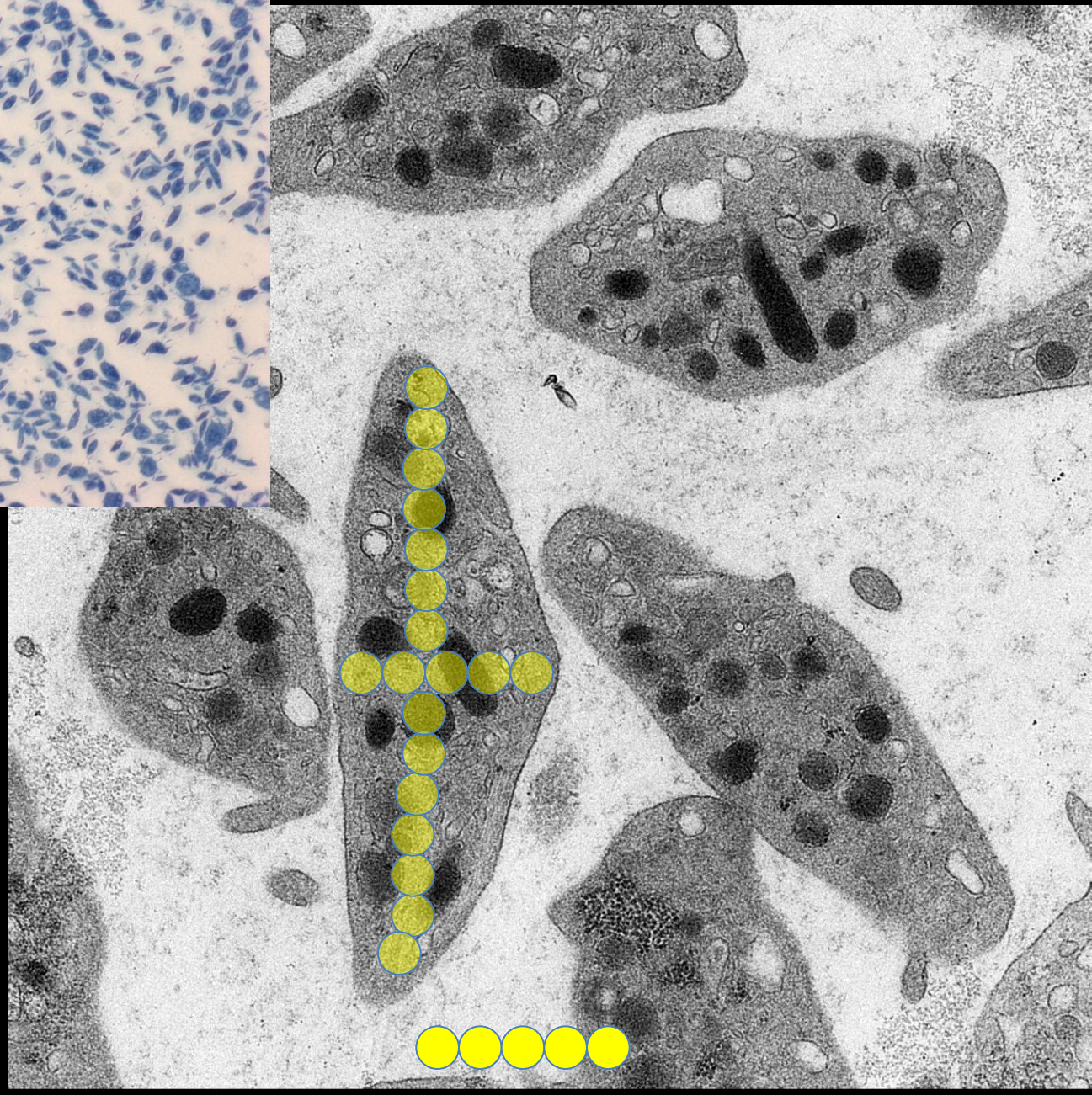

Ca. 220 nm
PSF: XY projection
Ca. 600 nm
PSF: XZ or YZ projection
18 details across the nucleus
10µm
Less than 50 details on 10 microns



10 um

5 x 15 details for a platelet

Less than 2 for Z-dimension!



Light microscopy vs. Electron microscopy

Advantages:

1) Simple
2) Live cells
3) Large area
4) Multiple labeling

Disadvantages:

1) Diffraction-limited XY-resolution ca. 200 nm ≈ 50 lines for 10 um cell
2) Poor Z-resolution > 600nm mostly top vs. bottom
3) Poor recognition of organelles

Advantages:

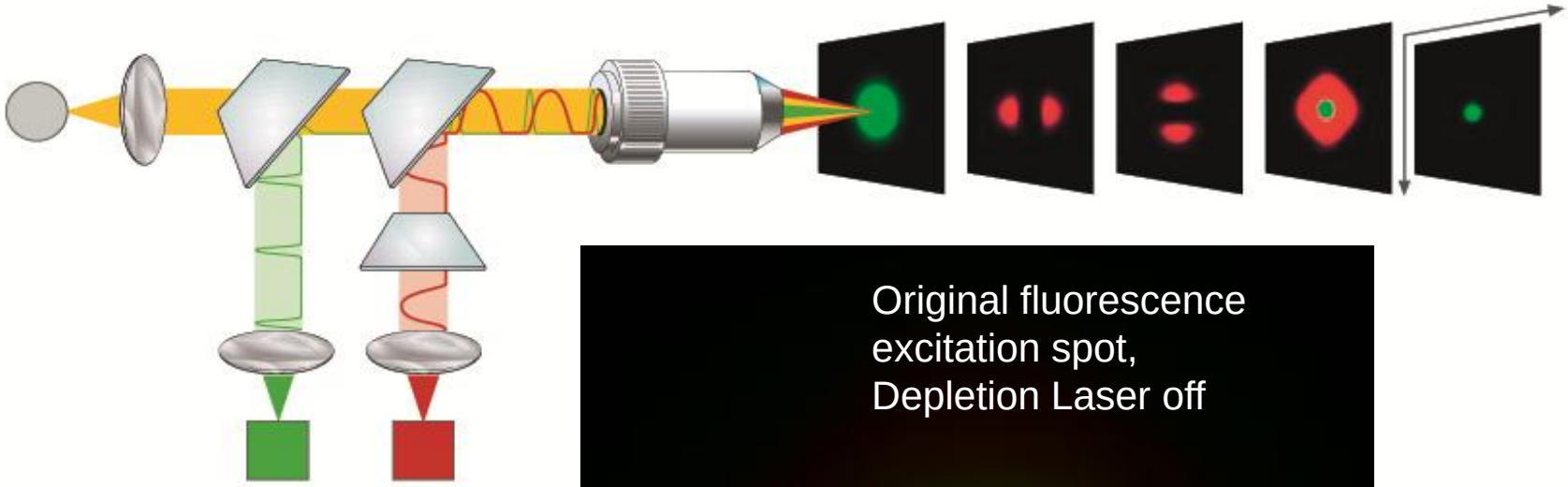
1) High resolution
2) Structural recognition

Disadvantages:

1) Vacuum kills: no live cells
2) Only thin sections or surface = limited info
3) Poor immunolabeling
4) Multiple labeling difficult
5) Expensive


Super-resolution or
correlative microscopy?

S T E D = STimulated Emission Depletion



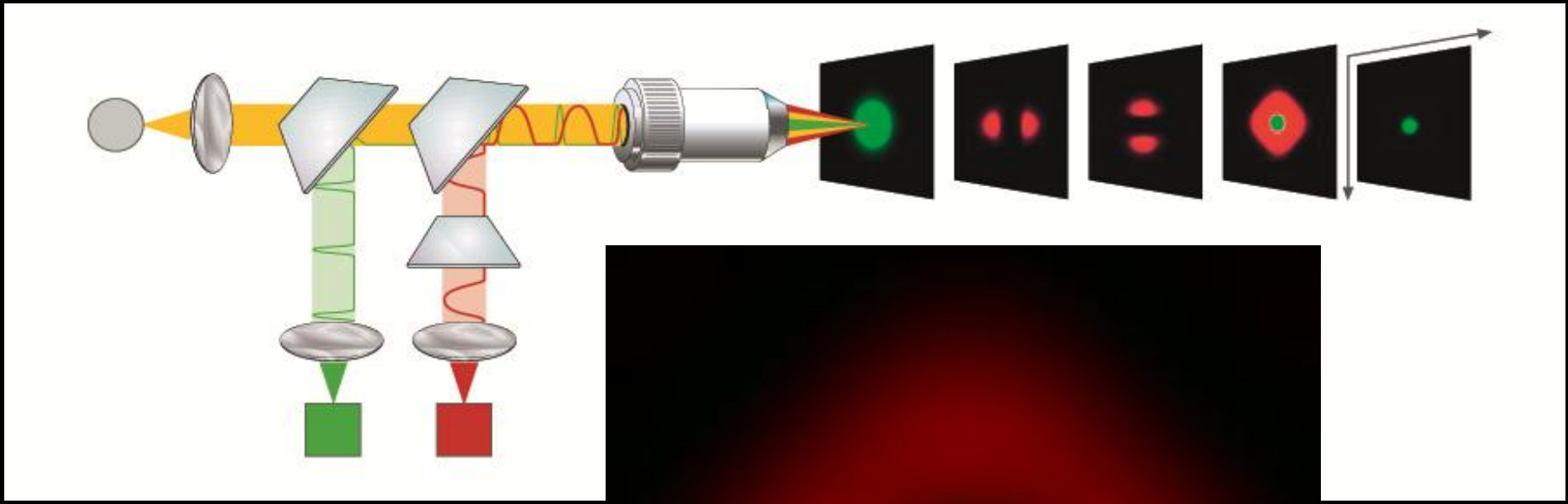
Original fluorescence excitation spot, Depletion Laser off

200 nm

Two superimposed beams:

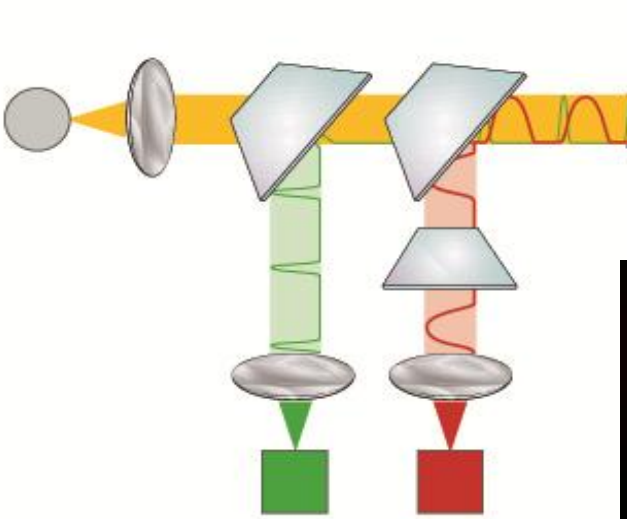
- Excitation laser Pulsed (<10ps) → 635nm
- Depletion laser: Pulsed donut-shaped red-shifted (IR, 200-300ps)
- Dyes that can be depleted effectively with low re-excitation → ATTO 647N or ATTO655

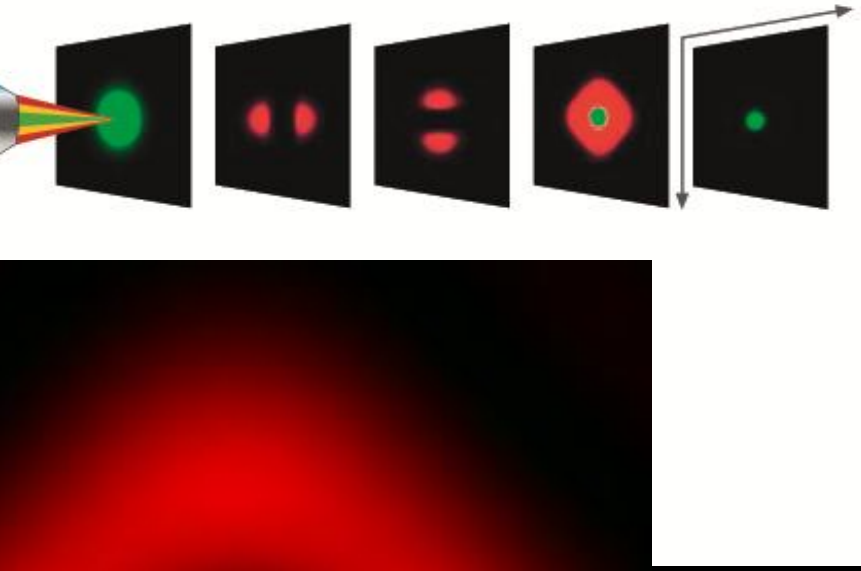
STED spot size reduction principle

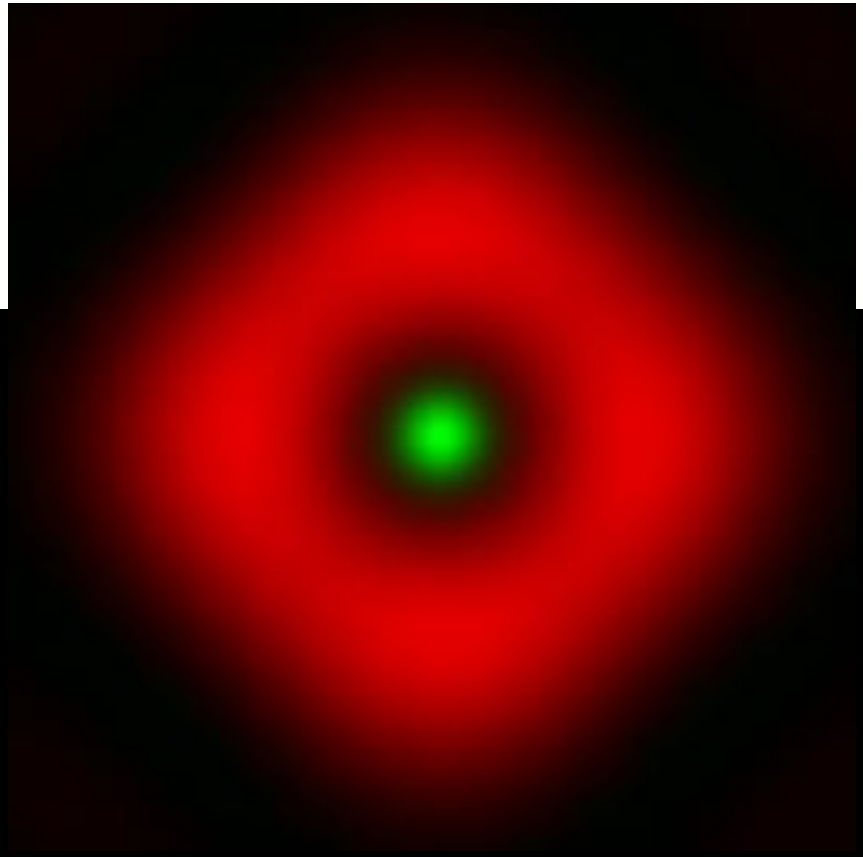


Depletion Power: 1

STED spot size reduction principle

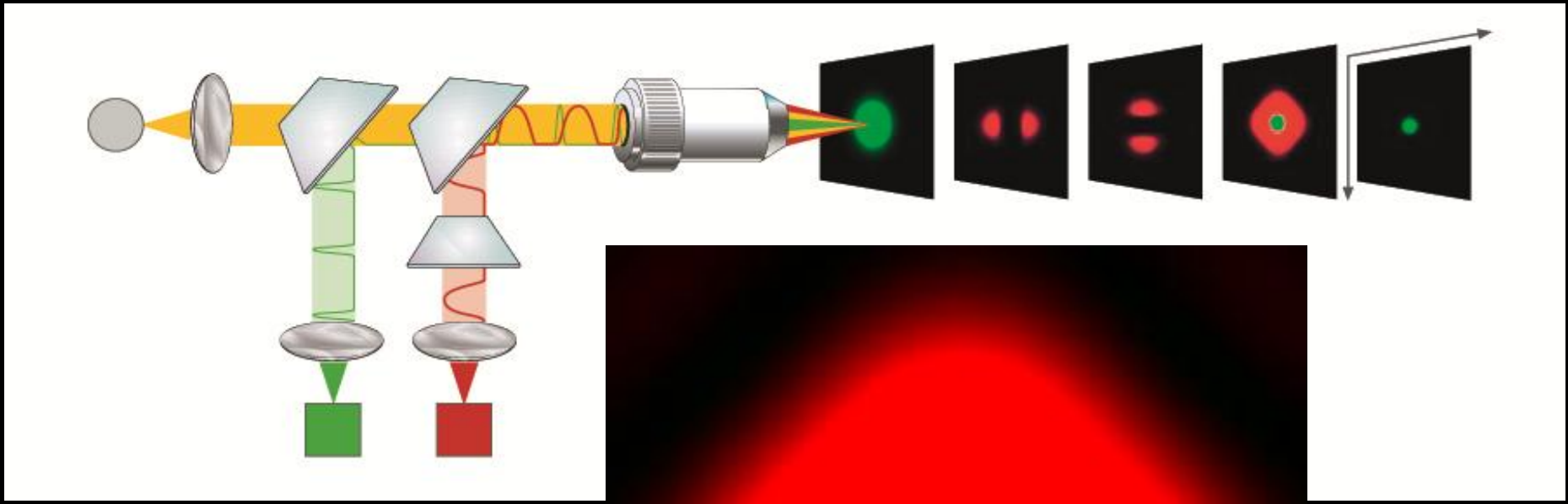


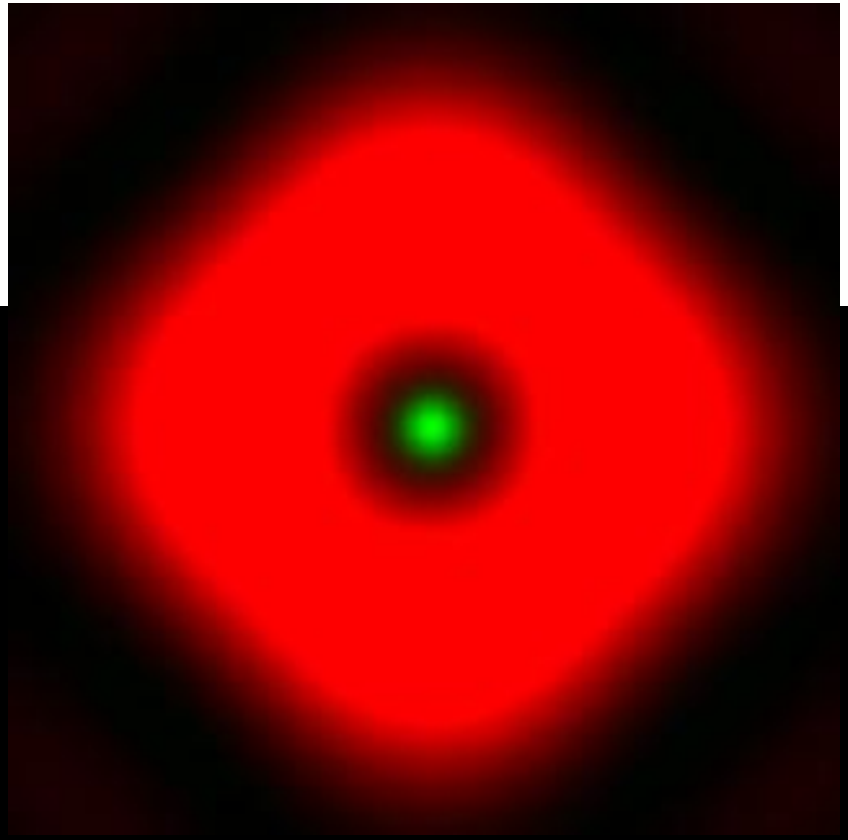




Depletion Power: 2

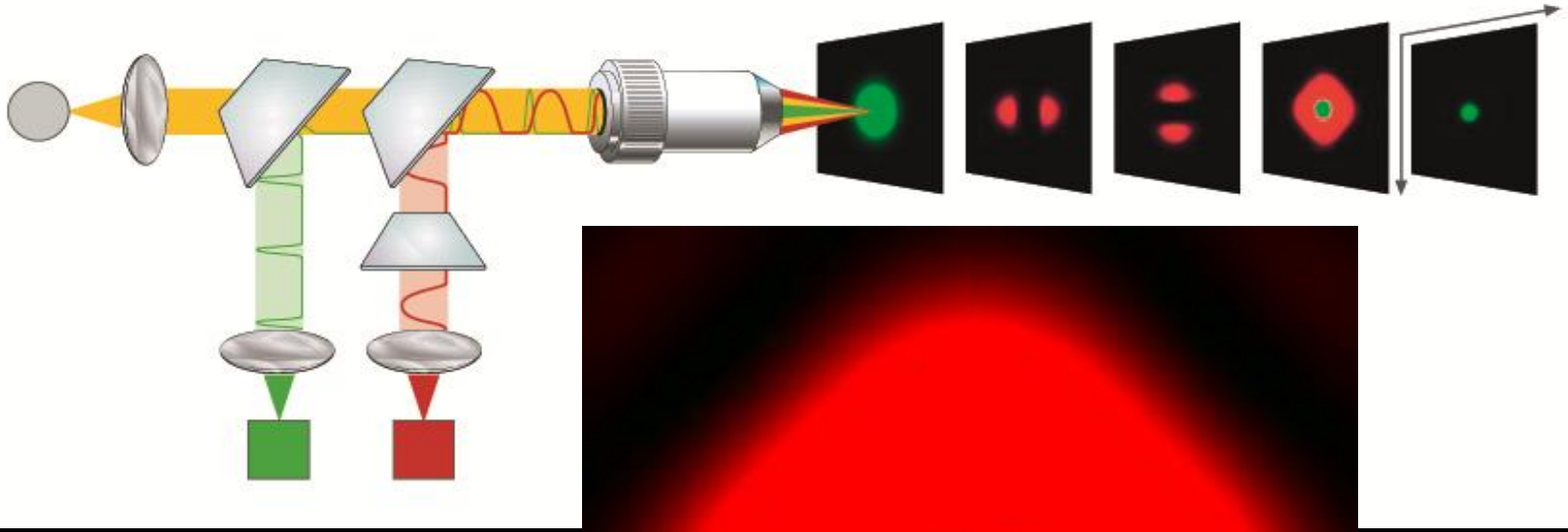
STED spot size reduction principle

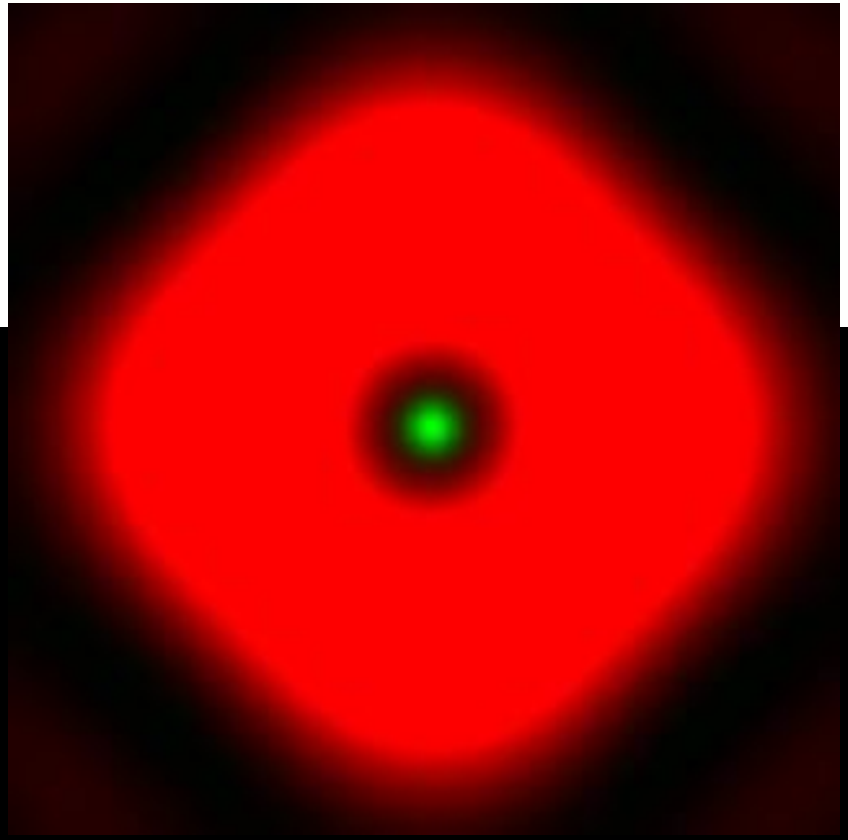




Depletion Power: 3

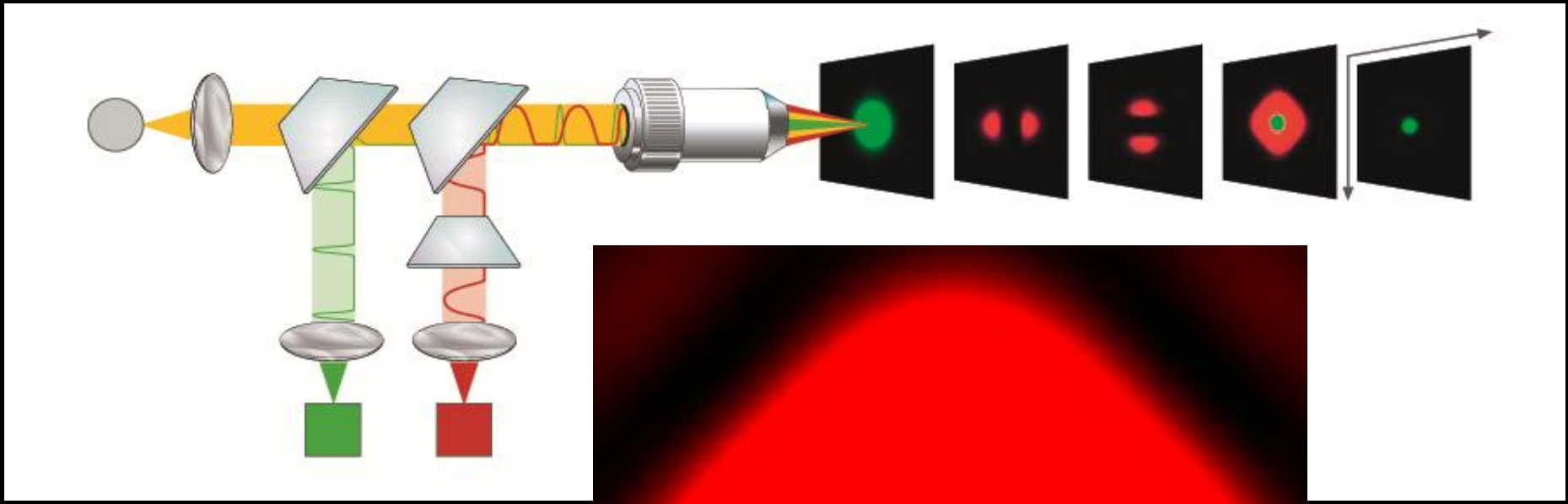
STED spot size reduction principle

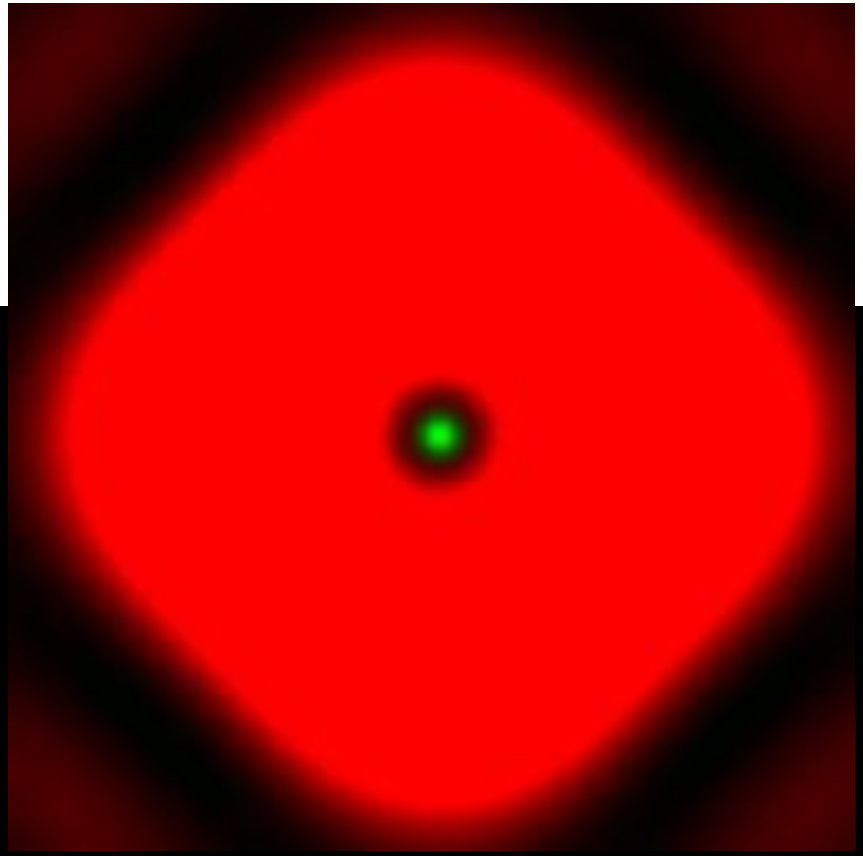




Depletion Power: 4

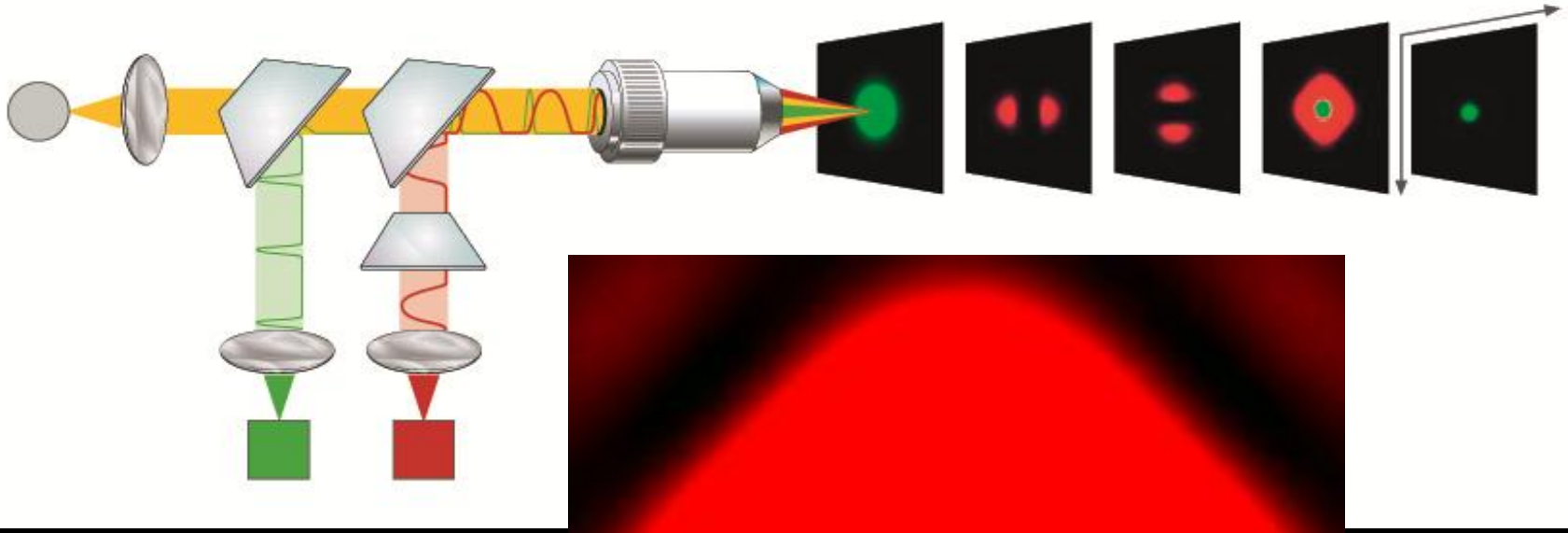
STED spot size reduction principle

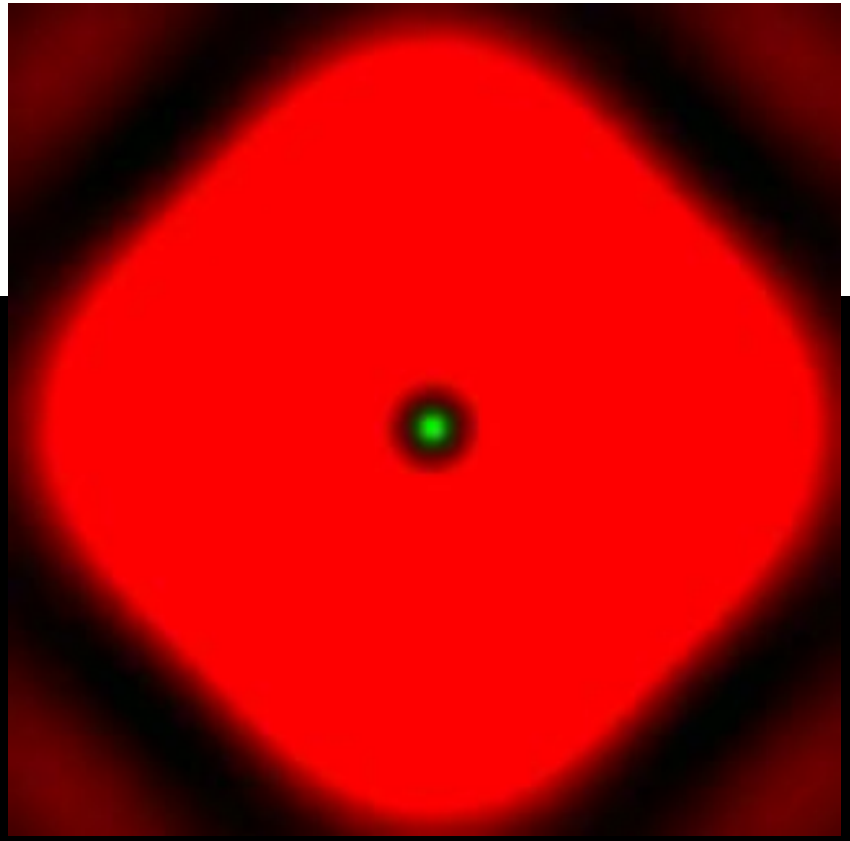




Depletion Power: 5

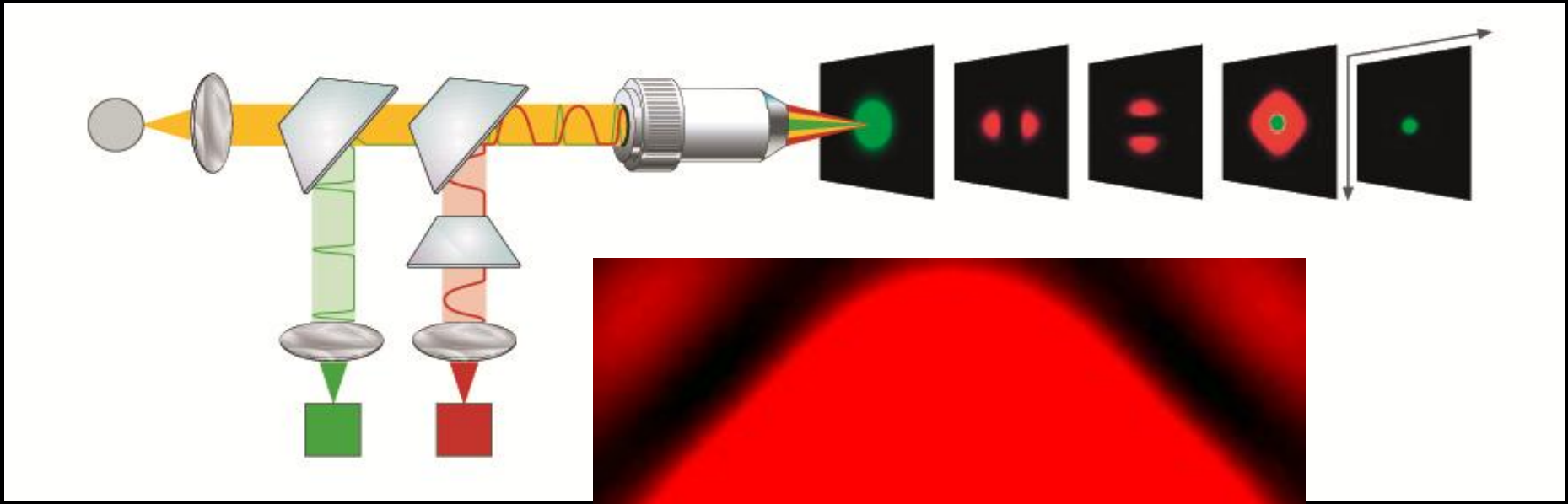
STED spot size reduction principle

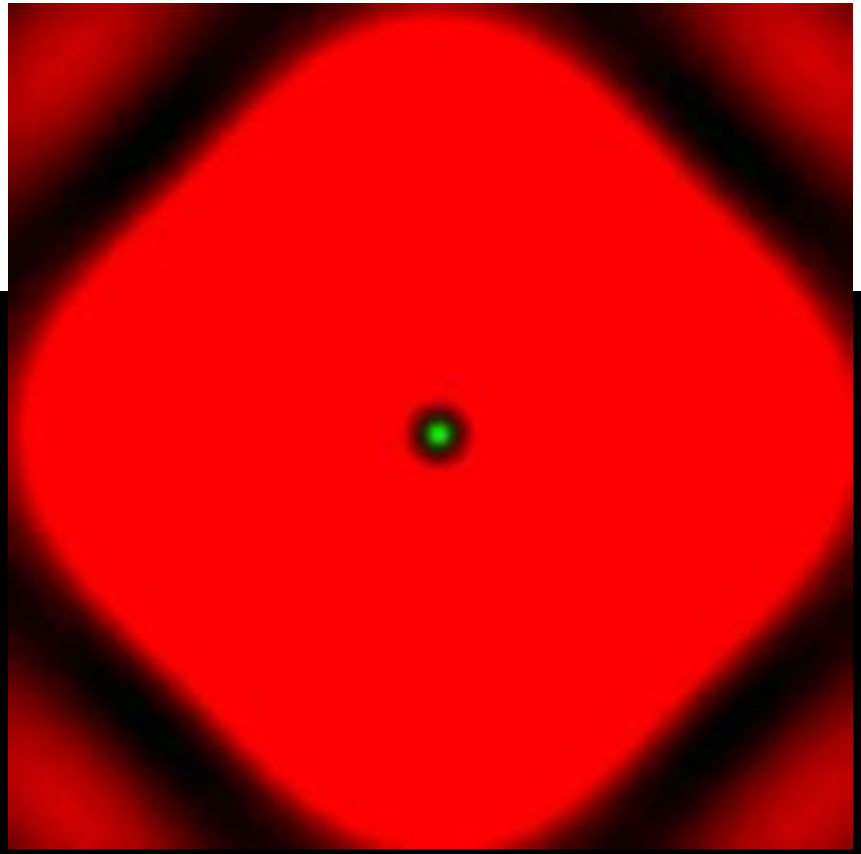




Depletion Power: 6

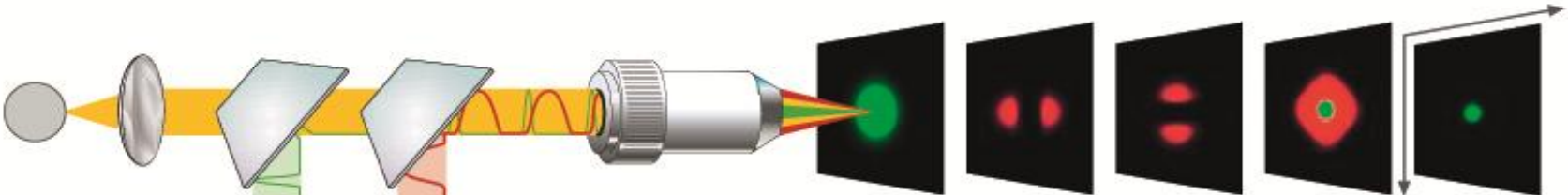
STED spot size reduction principle

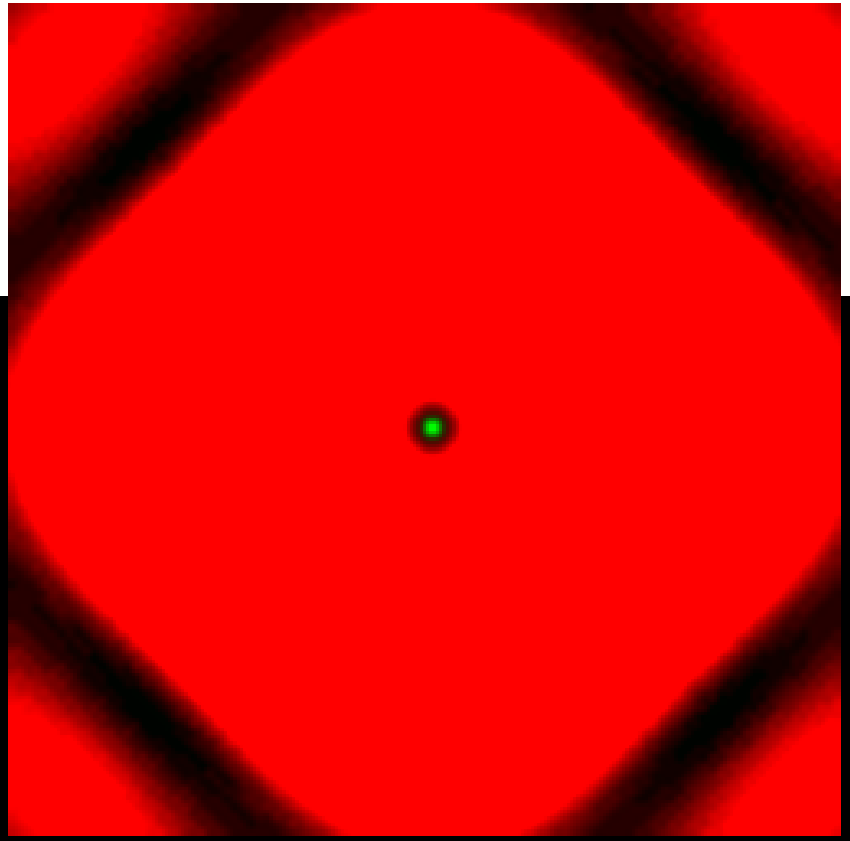




Depletion Power: 7

STED spot size reduction principle

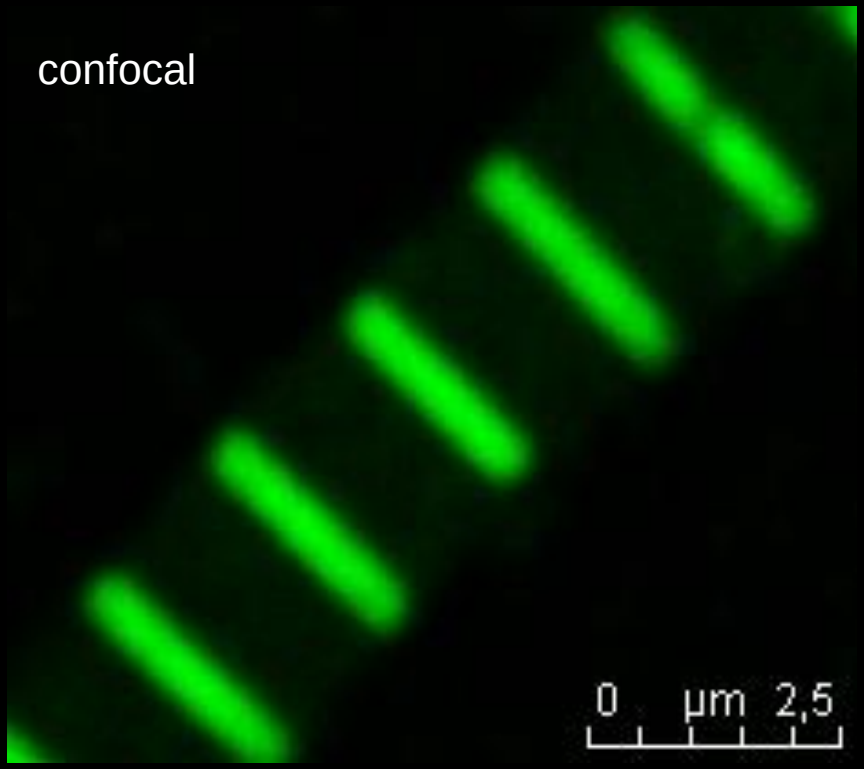


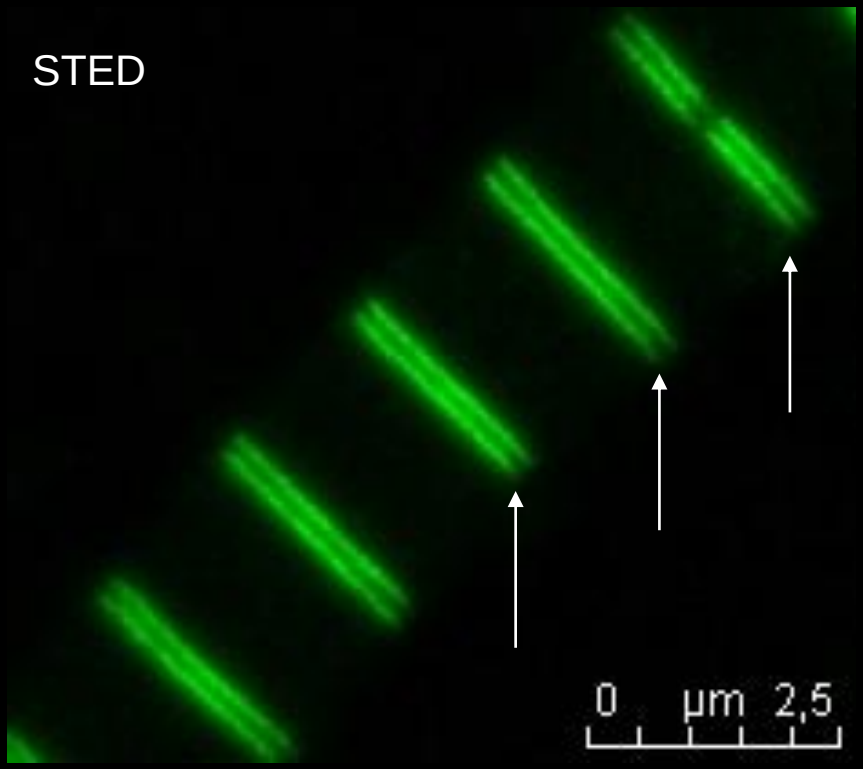


Depletion Power: 8

XY Resolution
in STED is mainly
determined by depletion
power

Myosin in Mausmuskelfaser





Superresolution: comparison

SIM

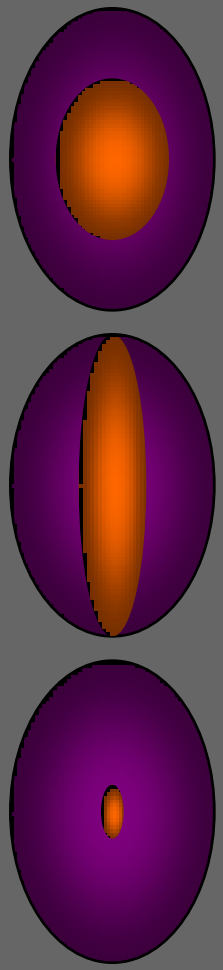
Structured Illumination Microscopy

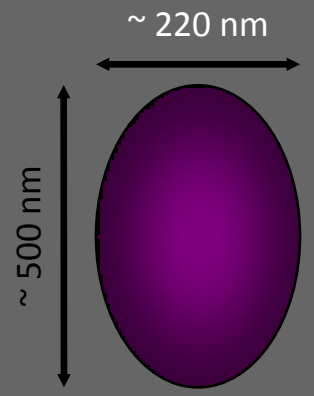
STED

Stimulated Emission Depletion

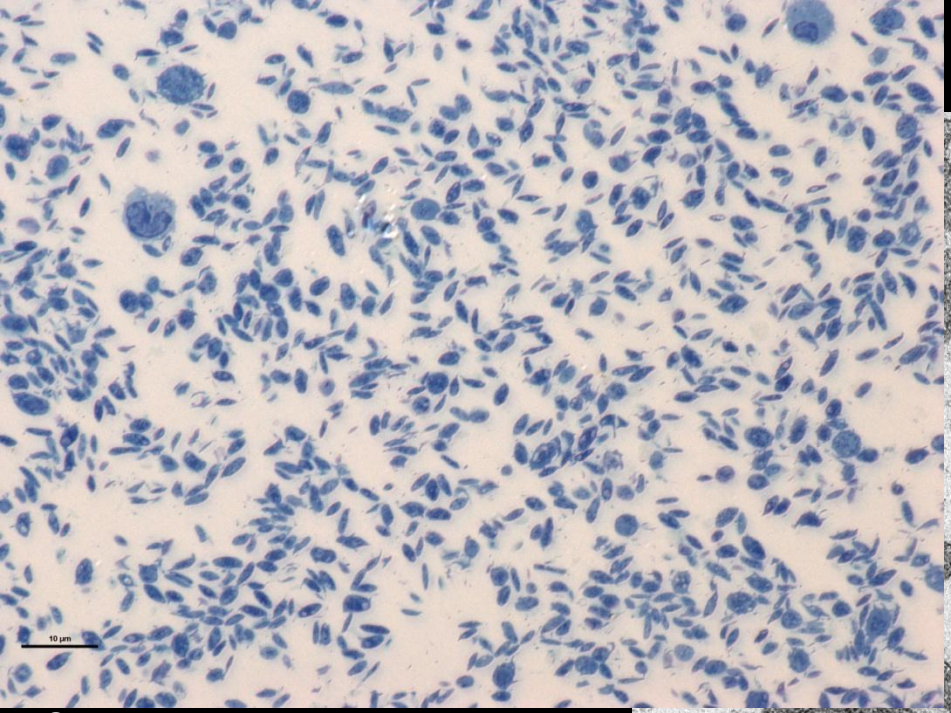
PAL-M

PhotoActivated Localization Microscopy





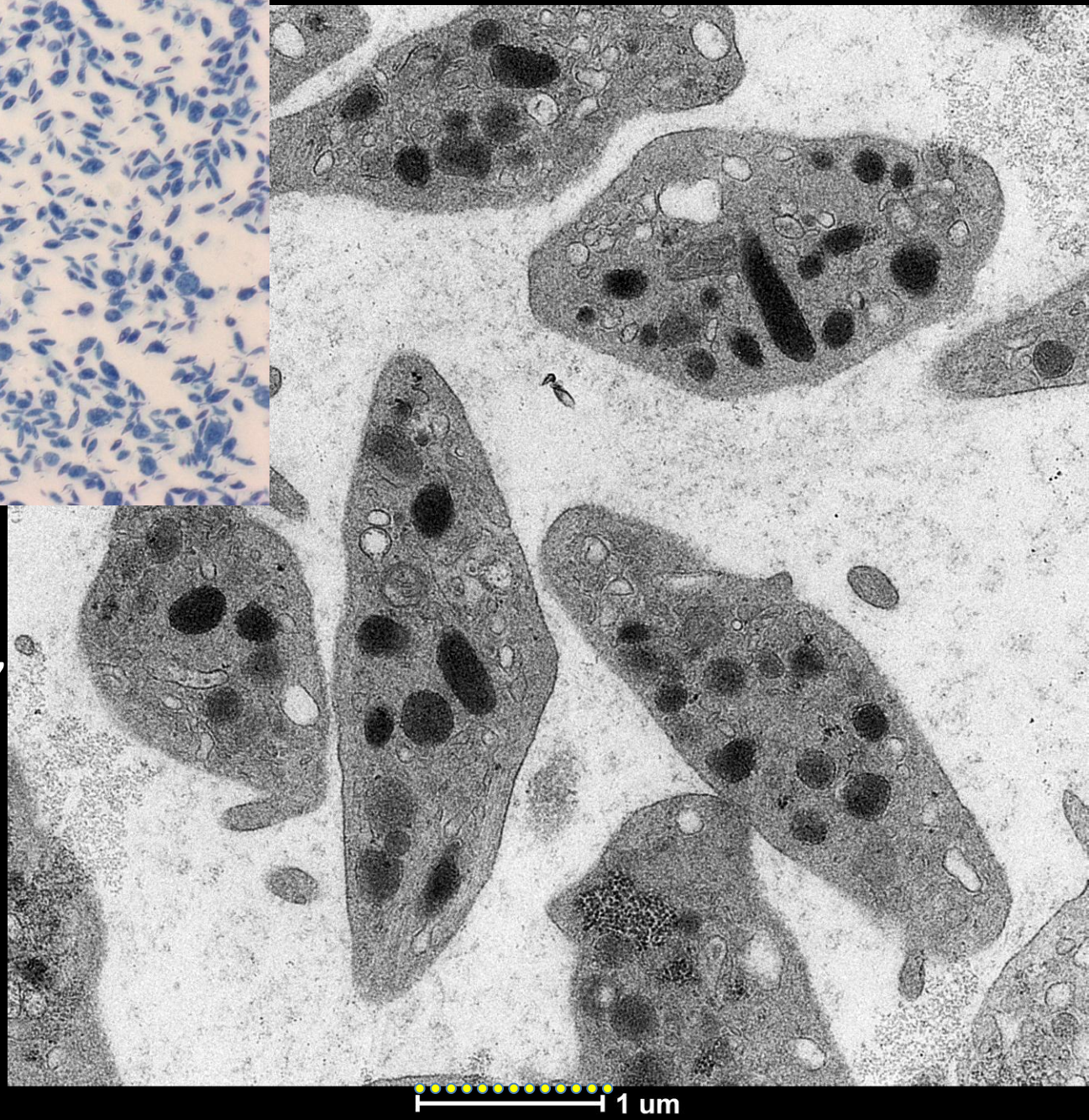
Confocal Resolution



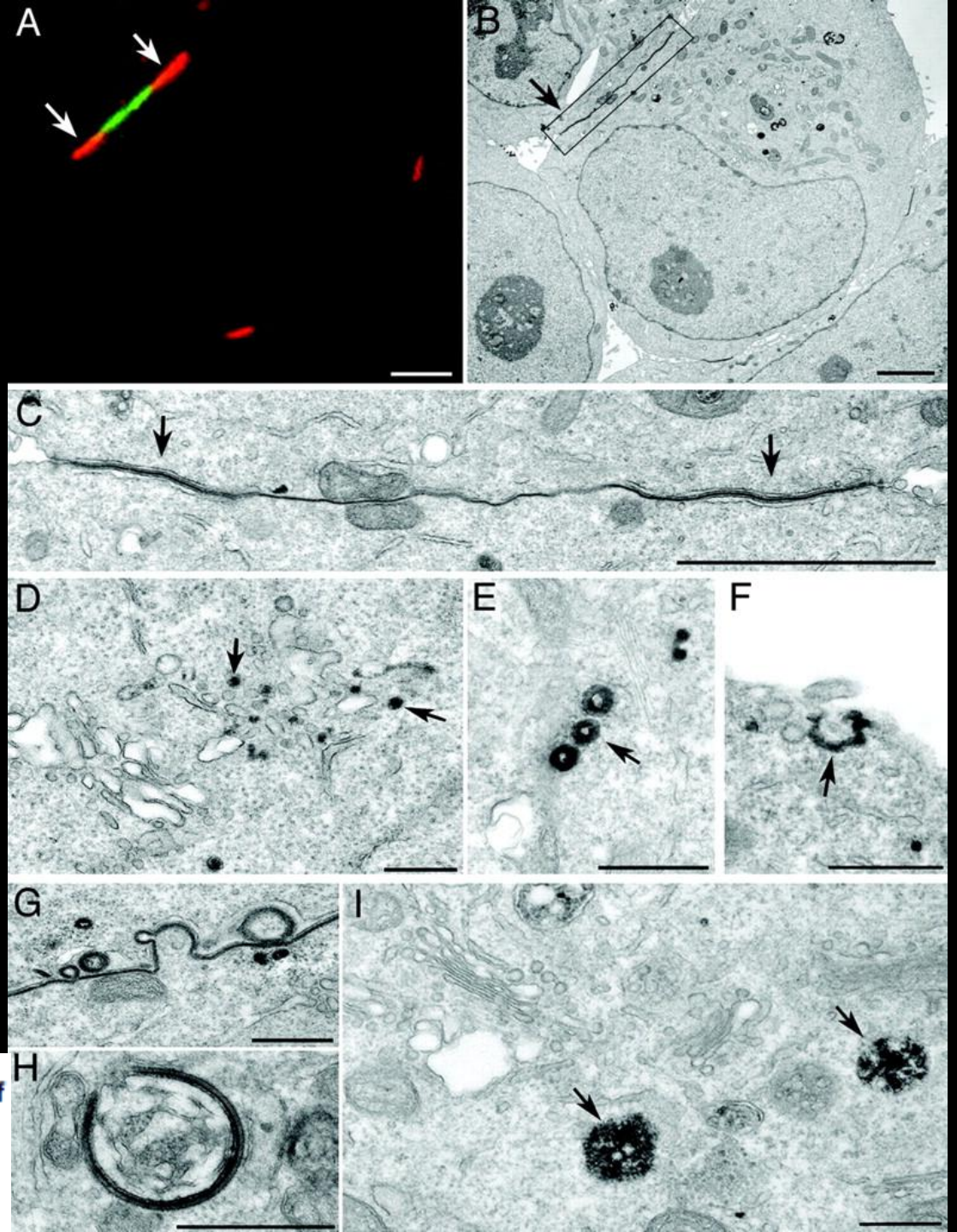
10 um

If 40 nm instead of 220,

Ca. 30 x 80 details for a platelet



C L E M =
Correlative
Light
Electron
Microscopy



Multicolor and Electron Microscopic Imaging of Connexin Trafficking
Guido Gaietta, *et al.*
Science **296**, 503 (2002);
DOI: 10.1126/science.1068793

C L E M = Correlative Light Electron Microscopy

Transfection Cx43-tetracisteine

Live cells: ReAsH (A)

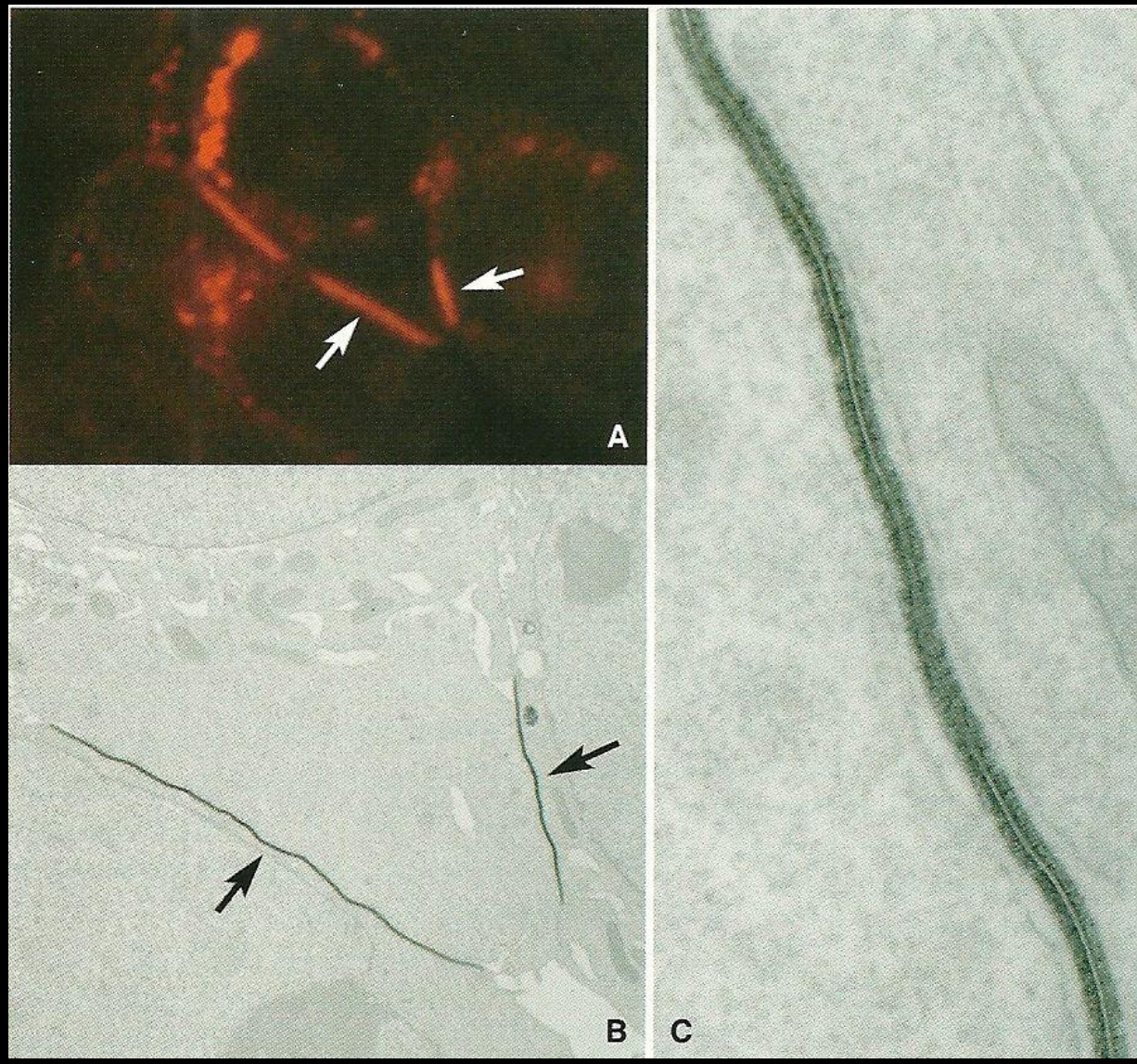
Fluorescent microscopy

Fixation

DAB+ UV O*

Epon

EM (B, C)



C L E M = Correlative Light Electron Microscopy

Fixierung

Anti-β-tubulin

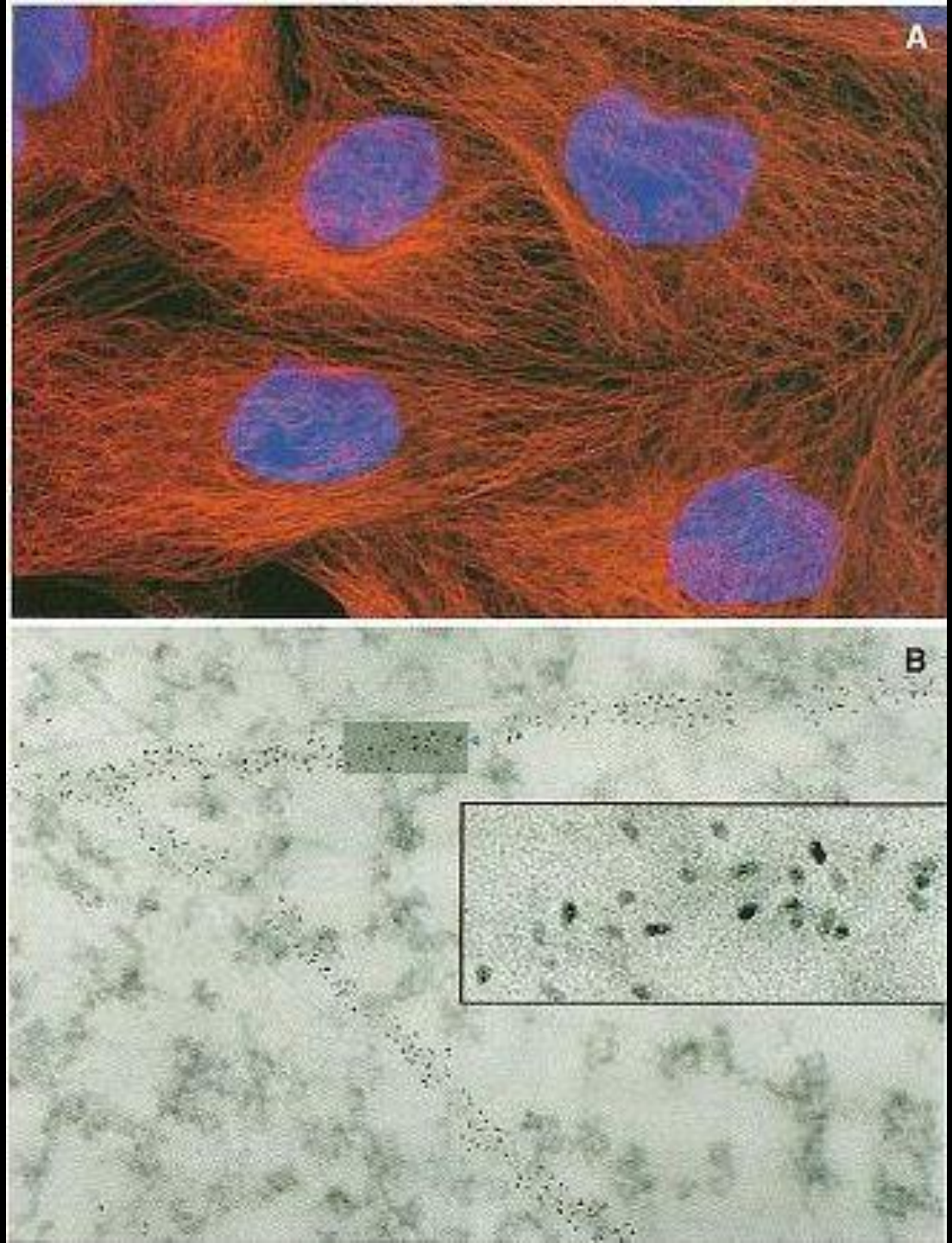
Secondary*quantum dot_655

DAPI

Fluoreszenzmikroskopie (A)

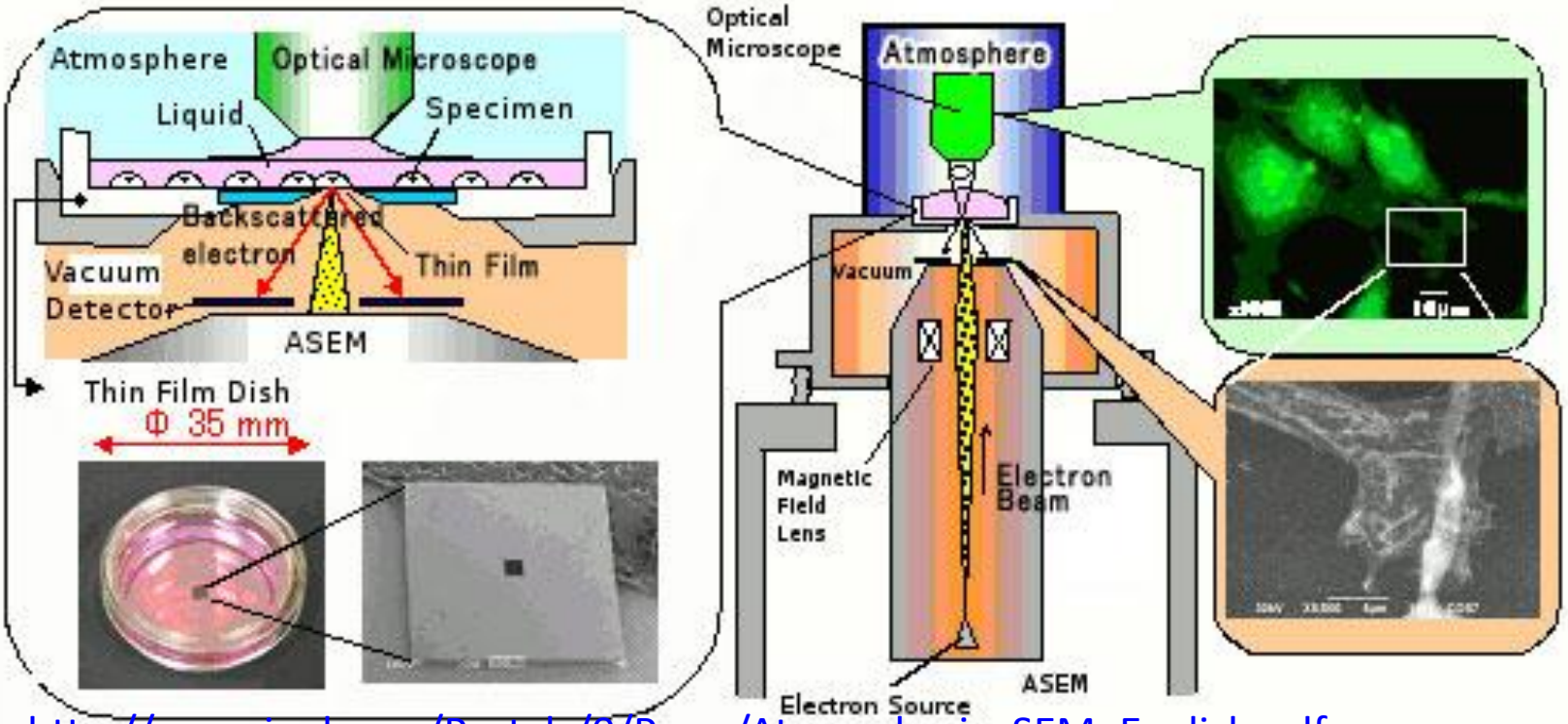
Fixierung - Epon

EM (B)



Up Fluorescence, d ownSEM

JEOL JASM-6200 Scanning Electron Microscope ClairScope 2010



http://www.jeol.com/Portals/0/Press/Atmospheric_SEM_English.pdf

We develop an X-ray-Fluorescence combination

	Practically achievable XY resolution, nm	Features, Limitations
Fluorescence microscopy, including confocal and multi-photon	200 nm	- simple, suitable for live cells - multiple labeling, large field of view - suitable for fast acquisition (30 fps and more) - low resolution
Electron microscopy	1 nm (bio-EM) 10 nm (immuno-EM)	- vacuum: unsuitable for most live cell studies - thin samples only (70-300nm): reduced information - expensive sample preparation - immunolabeling difficult and decreases the resolution
Super-resolution fluorescence: STORM, PALM, STED	40 nm	- slow: unsuitable for many live cell studies - require photo switchable fluorescent dyes or proteins
Near UV microscopy	100 nm	- requires special expensive lenses - poor signal/noise ratio - only 2-fold gain of resolution
EUV microscopy now	40 nm	- requires special light sources and X-ray optics - narrow field of view